CARDIOVASCULAR RISK FACTORS AND SUBCLINICAL ATHEROSCLEROSIS IN MIDDLE AGED WOMEN WITH A HISTORY OF POLYCYSTIC OVARY SYNDROME

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The final menstrual period defines menopause and signifies depletion of ovarian follicular reserve and endogenous estradiol. Diminished estradiol underscores postmenopausal increases in chronic health conditions of non-reproductive tissues namely the vascular, skeletal, and central nervous systems. Polycystic ovary syndrome (PCOS) is a prevalent disorder associated with an increased risk for type 2 diabetes and an adverse cardiovascular disease (CVD) risk profile evident at younger ages. It is well established that the risk of CVD increases among women following menopause. However, no definitive studies exist demonstrating increased cardiovascular morbidity or mortality among older women with a history of PCOS. Further, the association between menopause and CVD risk factors has not been fully explored in women with a history of PCOS. Women with PCOS report less menstrual cycle irregularity across time that may reflect varying degrees of ovarian function that in turn may augment CVD risk factors. We evaluated the hypotheses that menopause and lifetime menstrual cycle irregularity would have a modifying effect on CVD risk factors and markers of subclinical atherosclerosis in 152 women with PCOS and 169 normal reproductive controls ages 35 to 67 years. We found that the typical reproductive presentation and the adverse lipid profile observed in younger PCOS women was not as apparent in older PCOS cases compared to controls. Twenty-five percent of menopausal cases, however had type 2 diabetes. Coronary artery calcification (CAC) was greater in cases compared to controls and increased with age. PCOS cases reporting the greatest menstrual irregularity across time had higher total and free testosterone levels and greater CAC compared to cases with more frequent cycles. Our studies support the importance of diabetes prevention in aging women with a history of PCOS to reduce risk for early cardiovascular disease. Further, women with PCOS who present with the greater cycle irregularity may be more likely to have cardiovascular consequences. **Relevance to Public Health:** Because preventing PCOS is unlikely, interventions focused on promoting healthy aging among women with a history of the condition represents an important undertaking that will temper long-term health burden and improve quality of life.

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PREFACE

Reflecting on my professional experiences and the bumps and stalls that occurred on the protracted course of my graduate studies, I have come to appreciate the lessons of each and realize those obstacles served to preserve my career pathway in women's health research; an area that has been personally rewarding, intellectually stimulating, and appreciably worthwhile.

Many individuals influenced and encouraged me during my doctoral training and merit recognition and my gratitude. First and foremost they are: my children, Lindsey and Zachery Daniels, sources of inspiration and reminders of the truly important things in life; Scott Thomaston, infallible in his encouragement and understanding; my parents for modeling a strong work ethic and sense of responsibility and encouraging my independence; Lyn Cost whose hospitality and friendship I hold dear; Elizabeth Shire for imparting her passion for scientific inquiry and lifelong learning; friends, colleagues, and mentors in the Department of Gynecology and Obstetrics at Emory and especially, Ira Horowitz, Georgia Brogdon, Lisa Haddad, and Melissa Kottke for the sponsorship, coaching, opportunity, and goodwill they expressly contributed; Sarah Berga who introduced me to clinical investigation and stipulated an environment that allowed me to develop beyond my job title and position; the members of my committee for providing thoughtful mentorship, direction, and support in the execution of this dissertation; and lastly, Evelyn Talbott for suggesting during a CHARM I working group that I sign up for an Epidemiology class. She acquainted me with an alternate pathway, and I am indebted to her for encouraging me to consider the field of Public Health. I look forward to applying the knowledge and expertise gained in graduate preparation as I continue to develop as a researcher and advocate for women's reproductive health.

1.0 INTRODUCTION

Polycystic ovary syndrome (PCOS) manifests as anovulatory infertility with androgen excess and predisposes metabolic syndrome. These features coexist to varying degrees resulting in a spectrum of reproductive, endocrine, and metabolic presentations that affect a significant proportion of women. PCOS increases the risk for type 2 diabetes and is associated with adverse cardiovascular disease (CVD) risk profiles as well as evidence of subclinical atherosclerosis at earlier ages(1, 2). The etiology of PCOS remains undefined despite considerable research. Variants in genes regulating steroid biosynthesis, insulin receptor, and follistatin have been associated with PCOS in some studies however it is generally agreed that complex gene-environment interactions likely influence the spectrum of clinical presentation(3, 4). Treatments focus on the improvement of the patient's presenting symptom(s) and may include oral contraceptives to regulate menstrual cycles, ovulation induction when pregnancy is desired, and behavioral, pharmacological and/or medical interventions to address metabolic symptoms(5).

In spite of the strong association between PCOS and metabolic syndrome, definitive evidence of an increased rate of morbidity or mortality due to cardiovascular events does not exist. Advancing chronologic age has been associated with an increase in menstrual cycle frequency (6) accompanied by a decrease in androgen levels (7) among women with PCOS. Compared to women with normal reproductive histories, those with PCOS possess greater numbers of primary ovarian follicles (8) that remain viable and responsive to stimulation with advancing age (9). Together these observations suggest a normalization of reproductive function with age in PCOS. This notion is intriguing in the context of long-term health risks; for it is known that women who experience premature or early menopause are observed to be at increased risk of early cardiovascular, skeletal, psychological, and neurological morbidity and

mortality (10). Moreover, women who experience rapid transition to menopause demonstrate accelerated progression of preclinical CVD indicators compared to women with a slower menopause transition (11). There is however a paucity of information about the menopause transition in women with PCOS and how menopause effects CVD risk in this population. Reasons for this gap include the relatively recent availability of diagnostic and research tools necessary to evaluate endocrine and reproductive function, inconsistency with regard to the definition of PCOS and menopause, and the limited number of longitudinal studies following women with a history of PCOS through middle and older age.

The significance of this knowledge gap was recognized by the European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM) during the 2010 Amsterdam PCOS consensus meeting. Published in 2012, the consensus report identified several areas for future inquiry related to PCOS and menopause including, the evaluation of the age of menopause, characterization of the PCOS menopause phenotype, and the initiation of multi-center, longitudinal cohort studies to assess the menopause phenotype as well as long-term risk of morbidity and mortality among women with a history of PCOS (12). The Cardiovascular Health and Risk Measurement (CHARM) study directed by Dr. Evelyn Talbott began as a matched case-control study aimed at evaluating CVD risk factors in women with documented PCOS and their normal reproductive control counterparts during the middle to late reproductive years. A significant proportion of the participants were re-evaluated after 10 years during CHARM phase III. Our study examines the association between PCOS and menopause on CVD risk factors and subclinical atherosclerosis in PCOS cases and controls evaluated in their late reproductive to early menopausal years during CHARM-III.

1.1 Hypothesis and Specific Aims

Young women with PCOS have increased CVD risk factors and subclinical atherosclerosis and it is well known that the risk of CVD increases among all women following menopause. The association between menopause and cardiovascular disease risk factors has not been fully explored in older women with a history of PCOS. Further, women with PCOS report varying degrees of menstrual cycle irregularity that may reflect varying degrees of ovarian function, (estrogen exposure) that in turn may augment cardiovascular risk factors. Recently it was shown that the decrease in estradiol and stable androgen levels associated with the menopausal transition in women with normal reproductive history led to a state of relative androgen excess that resulted in an increase risk for metabolic syndrome (13). Conversely, among women with PCOS there is evidence indicating that androgen levels decline and menstrual cycles become more frequent with age. Women with PCOS also display elevated ovarian anti-müllerian hormone (AMH) levels compared to normal reproductive women. Both AMH and estradiol are produced by granulosa cells of the ovarian follicle. To date the relationship between AMH level and androgen to estradiol ratio in an older reproductive age group of women with a history of PCOS has not been documented. This investigation will examine the associations between PCOS, menopause, menstrual irregularity, and CVD risk factors and subclinical atherosclerosis in a cohort of middle adult women who participated in the Cardiovascular Health and Risk Measurement III (CHARM-III) study.

Specific Aim 1: examine the association between menopause and CVD risk factors and subclinical atherosclerosis (SCA) as determined by coronary artery calcification (CAC) in middle-aged women with a history of PCOS and controls adjusting for age, body mass index, as well as other known predictors of CVD risk factors.

Hypothesis 1: menopause will further exacerbate risk factors for CVD and subclinical SCA in older women with a history of PCOS compared to controls.

Specific Aim 2: examine the association between degree of menstrual irregularity, determined by number of cycles reported across earlier reproductive years, and CVD risk factors and CAC among women with PCOS who were evaluated during later reproductive to menopausal years.
Hypothesis 2: greater menstrual irregularity across time will be associated with a more adverse CVD risk profile and SCA documented by CAC in women with a history of PCOS.

Specific Aim 3: examine the relationship between ovarian reserve, documented by anti-müllerian hormone (AMH) level and relative testosterone to estradiol ratio, documented by the ratio of free androgen index to free estradiol index (FAI/FEI) in late reproductive age women with PCOS compared to controls.
Hypothesis 3: decreases in AMH level will be associated with an increased FAI/FEI ratio in both PCOS and controls. PCOS will have a smaller change in FAI/FEI that correlates with a higher AMH level.

To summarize, PCOS is associated with an increased the risk for chronic health conditions that extend well beyond the reproductive portion of a woman's life. Limited data is available on the relationship between PCOS, menopause, and risk factors for cardiovascular disease. Preventing PCOS is unlikely, thus, developing strategies to facilitate healthy aging among women with the condition represents an important undertaking that will contribute to a reduction of long-term health burden. The information garnered from this investigation may result in better strategies for management of PCOS through the menopause transition and ultimately improve health and quality of life for those with the disorder.

2.0 BACKGROUND AND RATIONALE

This section provides a: 1) general overview of the history, definitions, and prevalence of PCOS; 2) description of the reproductive and metabolic attributes of PCOS; 3) review of the physiological processes that define normal reproductive function and the menopause transition; 4) summary of our current understanding of aging in women with PCOS; and 5) framework for the proposed studies to evaluate the association between menopause, lifetime cycle irregularity and risk factors for cardiovascular disease and subclinical atherosclerosis in women with a history of PCOS.

2.1 Polycystic Ovary Syndrome: History, Definition, and Prevalence

In 1935, surgeons Stein and Leventhal published a case series describing the appearance of enlarged ovaries containing multiple small cysts in a group of amenorrheic, hirsute, women undergoing wedge resection (removal of a section of ovary). They observed that wedge resection was followed by restoration of menstrual cyclicity in these patients (14). Advances in laboratory medicine and medical imaging have since enabled physicians and scientists to further characterize the endocrine, metabolic, genetic, and morphologic features of this presentation; a condition now referred to as polycystic ovary syndrome (PCOS). PCOS is characterized by a range of clinical and endocrine features including, intermittent ovulation and menses, excess body hair, acne, central obesity, elevated serum levels of ovarian androgens and pituitary luteinizing hormone (LH), insulin resistance, and ultrasonographic appearance of polycystic ovaries. Heterogeneity in clinical presentation contributed to inconsistencies in the diagnosis

and definition of PCOS that hampered evaluation and comparison of studies published in the early literature.

Demands from the scientific and medical communities for uniformity in the definition of PCOS led to a 1990 National Institutes of Health (NIH) conference to develop criteria for the classification of PCOS. Although a consensus was not reached, it was generally agreed that PCOS would be defined as chronic oligo- or anovulation accompanied by clinical and/or biochemical evidence of ovarian hyperandrogenism that was exclusive of other causes such as congenital adrenal hyperplasia or androgen secreting tumors (15). Attendees were divided on ovarian ultrasonographic findings. Because polycystic ovaries had been observed in some women with normal reproductive function (16) and had not been observed in all women with PCOS (17) the appearance of polycystic ovaries on ultrasound was not deemed necessary for the diagnosis of PCOS.

In 2003 ESHRE/ASRM held a conference in Rotterdam and ratified an expanded definition of PCOS to include ovulatory and anovulatory women with polycystic appearing ovaries and normal androgen levels (18). Women with these presentations would not have met criteria for PCOS using the classic NIH definition. Many experts viewed the new definition premature voicing concerns about the integrity of future research, clinical management, and patient insurability (19) if women with polycystic ovaries who did not also display androgen excess or ovulatory dysfunction were now considered to have PCOS. Evidence in support of both the exclusion and inclusion of these presentations can be found in the literature. Johnstone et al studied 262 ovulatory women and found that 32% of the subjects had evidence of polycystic ovaries on ultrasound but there was no association with metabolic perturbations typically observed in women with PCOS (20). Conversely, the fertile sisters of infertile women with PCOS often have some but not all of the features associated with classic PCOS and may be considered a reflection of the full spectrum of PCOS presentations (21). The Androgen Excess and PCOS (AE-PCOS) Society organized a taskforce to reconcile the NIH and Rotterdam definitions and

published a blended definition in 2009. The AE-PCOS defined PCOS as hyperandrogenemia (clinical and/or biochemical) and ovarian dysfunction (oligo-anovulation and/or polycystic ovaries) with other causes of androgen excess excluded (22) (23). **Table 1**, contrasts the NIH, Rotterdam, and AE-PCOS definitions for ten possible combinations of PCOS features.

Using the classic NIH definition, PCOS affects between 4% and 11% of all women (24). The prevalence increases 1.5 fold when the Rotterdam criteria are applied (25). PCOS is the underlying cause of infertility in 30% of women seeking evaluation for anovulation (26) and is present in 82% of women seeking evaluation for androgen excess (27). Carmina et al, evaluated 950 women with androgen excess using the Rotterdam criteria and found classic anovulatory PCOS in 60% and ovulatory PCOS in 15% of participants (28). Several teams have examined the degree of metabolic derangement across PCOS phenotypes and there is agreement that classic PCOS (hyperandrogenism combined with anovulation) is associated with more adverse metabolic and cardiovascular risk profiles compared to more subtle PCOS types (29-31). The overwhelming majority of women with PCOS (75%) meet the classic NIH definition (2).

Although PCOS is not isolated to any one particular race or ethnicity, evidence supports variability in the clinical phenotypic presentation between racial and ethnic groups. Lo et al, evaluated the cardiovascular risk profiles in 11,000 women diagnosed with PCOS in the Kaiser Permanente of Northern California health system. She found that women with PCOS of Asian descent were less likely and PCOS women of African or Hispanic origin were more likely to be obese compared to Caucasian women with PCOS. In addition, Asian and Hispanic women with PCOS were more likely to have diabetes (OR 2.16, 95% CI 1.63-2.85 and OR 1.33, 95% CI 1.03-1.71) compared to Caucasian women with PCOS. African American participants with PCOS were more likely to have hypertension (OR 1.32, 95% CI, 1.19-1.48) compared to PCOS cases of other races and ethnic groups (32). Using the NIH criteria, Welt et al studied 105 women with PCOS from Iceland and 262 women with PCOS from Boston and found that hormone levels (androgens and gonadotropins) and Ferriman and Galway scores (measure of hirsutism) varied

between ethnic groups but glucose and insulin levels were conserved across groups when adjusted for BMI (33). *Together, these studies reinforce the concept that PCOS is a common, multifaceted, chronic condition that affects women across all races and ethnicities with implications for negative health consequences that surpass the reproductive portion of a woman's lifespan.*

2.2 Reproductive Attributes of PCOS

Normal ovarian function results when neurons of the hypothalamus operate in synchrony releasing pulsatile bursts of gonadotropin releasing hormone (GnRH) that stimulate the anterior pituitary to secrete luteinizing hormone (LH) and follicle stimulating hormone (FSH). During the early follicular phase, FSH exceeds LH causing primary ovarian follicles to mature. Later, LH secretion surpasses FSH and a dominant follicle emerges. As the follicle matures it produces increasing amounts of estradiol (E2). Roughly 14 days from the onset of the last menstrual period, LH and E2 surge and a mature oocyte is released from the dominant follicle. Pulsatile release of LH and FSH slows and levels fall as residual follicular cells (corpus luteum) secrete progesterone and a second wave of E2 preparing the uterus to receive a fertilized egg. In the absence of conception, progesterone peaks around day 21 and falls rapidly resulting in endometrial shedding (menses) and the beginning of the next cycle (34, 35).

The first menstrual period defines menarche and denotes the pubertal transition. The median age of menarche has held relatively constant in recent history at 12.43 years. Females of African and Hispanic decent experience the first menstrual period at slightly earlier ages, 12.06 and 12.25 years respectively and girls with higher BMI also tend to experience earlier pubertal development (36). Menstrual onset does not however equate reproductive competence; rather a number of years may pass before the reproductive axis fully matures to support cyclic ovulation (36, 37). For this reason the diagnosis of irregular menses in the adolescent population is challenging.

Irregular menstrual cycles with intermittent ovulation is a cardinal feature of PCOS. Women with PCOS display increased pulsatile secretion of luteinizing hormone (LH) (38) that is resistant to feedback suppression by sex steroids (39). LH levels consistently exceed (FSH) levels as evidenced by a LH:FSH ratio greater than 1. Limited FSH stimulation is associated with the development of multiple small follicles rather than a dominant follicle, leading to the pattern of chronic anovulation and oligomenorrhea characteristic of PCOS. PCOS is often is detected in adolescence and patients with an early presentation are often at highest risk for metabolic complications (40).

Women with PCOS display evidence of increased ovarian androgen secretion. Normally, LH stimulates the cells of the follicle's outer theca layer to produce androstenedione, which is converted to testosterone and ultimately aromatized to estradiol by granulosa cells of the follicle's interior. Several potential explanations exist for ovarian androgen excess in PCOS including, 1) increased sensitivity of the theca to LH and insulin stimulation; 2) a decreased rate of conversion to E2 via aromatase activity in the granulosa cells; and 3) increased number of follicles contributing to the pool that is able to secrete androgens (41). Clinically androgen excess in PCOS is associated with acne, alopecia, and hirsutism. Testosterone levels in women with PCOS are typically elevated but lower than that observed in men, thus, significant virilization in the female patient is an indicator of a non-ovarian androgen source and warrants further evaluation (15, 18, 22).

Androgens produced by follicles of the PCOS ovary are converted to estradiol. Because ovulation does not regularly occur there is no luteal phase surge in progesterone to oppose this tonic level of estrogen. Untreated, unopposed estrogen exposure in women with PCOS contributes to the increased risk of endometrial carcinoma (42). Ovulation occurs spontaneously in PCOS and can also be achieved with correction of gonadotropin aberrations (43, 44). Other reproductive consequences of PCOS include ovarian hyperstimulation syndrome with ovulation induction, miscarriage, gestational diabetes, preeclampsia, and preterm birth with pregnancy.

Ovarian enlargement coupled with the appearance of multiple small follicles on ultrasound evaluation is a common feature of PCOS. During fetal development ovarian germ cells undergo mitotic replication; increasing in number until just prior to birth they enter meiosis forming primordial follicles, the basic functional units of the ovary. When mitotic replication ceases the ovary becomes endowed with a complete complement of follicles for life. Around the time of birth a significant proportion of this cohort is lost through atresia and growth of remaining follicles is arrested until puberty. Then with puberty follicles from the primordial pool enter the growing pool. A small number (roughly 500) of the estimated 2 million follicles endowed at birth, are destined for ovulation. Unselected follicles in the growing pool are eventually lost to atresia. Menopause ensues with depletion of the growing follicle pool.

The polycystic appearing ovary (PAO) is characterized by a thickened stromal core and increased number of small pre-ovulatory follicles (8). LH and insulin stimulation of the theca cells contribute to this observed thickening of the ovarian stroma and to the increase number of ovarian follicles with an increase in ovarian volume characteristic of the polycystic ovary (41). Possible explanations for the increased number of follicles in the ovaries of women with PCOS include a greater number of primordial follicles, increased entry of primordial follicles into the growing pool, and/or decreased clearance through atresia (45).

The evidence for increased numbers primordial in PCOS is inconsistent (8, 46, 47). However, there is agreement that the number primary growing follicles is greater in women with PCOS compared to those with normal reproductive history (46, 47). However, the explanation for this observation is less clear and may be due to an increased recruitment from the resting to the growing pool and/or a delay in clearance from the growing pool via apoptosis (atresia) (46, 48). Nevertheless, in vitro and in vivo studies demonstrate small arrested follicles of the PCOS ovary are responsive to appropriate hormonal stimulation (49) (50).

Biochemical evidence supports an increased follicle cohort in PCOS. Anti-müllerian hormone (AMH) belongs to the transforming growth factor beta (TGFβ) family of

cytokines, is produced by the granulosa cells of the developing follicle, and is believed to play a role in follicle selection (51). With increasing age, AMH decreases in direct correlation with declining follicle count and because levels are consistent across the menstrual interval it is preferred to inhibin B and FSH as a biochemical marker of ovarian reserve (51, 52). Elevated AMH levels are evident in women with PCOS from adolescence through the later reproductive years (53-55). Interestingly, in vitro studies have shown that this increase is not entirely due to an increase in follicle number rather granulose cells of the PCOS have higher rates of AMH production compared to cells from the ovaries of women with normal reproductive history (56).

2.3 Metabolic Attributes of PCOS

The coexistence of metabolic symptoms, hyperandrogenemia, and reproductive compromise was documented by Stein and Leventhal in their 1935 case series (14). In the years since that initial report, the metabolic sequelae associated with PCOS have been further characterized. Affected women frequently display increased central adiposity, obesity, hyperinsulinemia, type 2 diabetes, hypertension, an atherogenic lipid profile, an increase in inflammatory markers, and evidence of early subclinical atherosclerosis(57). The strong association with insulin resistance led to the inclusion of PCOS diagnosis as a major risk factor for the development of type 2 diabetes (1). Moreover, in 2010 the Endocrine Society in conjunction with the Androgen Excess and Polycystic Ovary Syndrome (AE-PCOS) Society commissioned an evidence-based review and evaluation of existing literature and development of guidelines for the prevention of cardiovascular disease specific to women with the condition (2).

Women with PCOS are at increased risk for developing type 2 diabetes. Insulin resistance independent of obesity is a cardinal feature of PCOS (58). Two independent investigations estimate the prevalence of insulin resistance to be between 60% and 80%, and

nearly all obese women with PCOS are also insulin resistant (59, 60). Impaired glucose tolerance affects between 30-35% of women with PCOS and approximately 10% will also have type 2 diabetes (61, 62). Recent studies suggest that 40% of women with PCOS will go on to develop pre- or frank diabetes by age 50 (63, 64). Obesity is a contributing factor, however even non-obese women with PCOS have abnormal glucose metabolism (58).

Women with PCOS have an adverse risk profile for cardiovascular disease. A number of reports documenting increased levels of CVD risk factors in women with PCOS compared to women with normal reproductive function have been published. Dahlgren et al, evaluated 33 women who underwent wedge resection two to three decades prior to evaluation and found an increased prevalence of diabetes and hypertension (65) and on further analysis calculated a 7 times greater risk for myocardial infarction in the PCOS cases compared to a group of 132 age matched controls (66). In another study of 102 women with PCOS and 19 lean normal reproductive controls, hypertension was observed in 6% of lean and 17% of obese PCOS subjects. This effect was only partially explained by insulin levels. Fasting glucose and androstenedione levels also contributed to the effect on blood pressure (67).

The Cardiovascular Health and Risk Measurement (CHARM) study, led by Dr. Talbott at the University of Pittsburgh represents one of the largest and well characterized prospective assessments of CVD risk among women with PCOS, **Table 2**, NIH R01HL4664-01. This investigation included 244 PCOS cases defined by the 1990 NIH criteria and 244 community, age, and race matched normal reproductive controls. The average age of women in CHARM I was 35 years and the cohort was predominantly white (92.4%). PCOS cases exhibited a more atherogenic risk profile that was evident in the premenopausal period (68) and independent of ultrasound ovarian appearance (69). PCOS predicted a more adverse lipid profile at younger ages when compared to control women (70). Middle-aged women with PCOS may be at risk for premature carotid atherosclerosis (71, 72), an observation that could not be fully explained by adjustment for body mass index (BMI). More recently, Talbott et al have shown that PCOS is

associated with increased calcification in the aorta and coronary arteries but the effect of PCOS is largely mitigated by the severity of indicators of the metabolic syndrome (73, 74).

Polycystic appearing ovaries are observed more often in women undergoing coronary angiography (75). However when Pierpoint et al, evaluated a group of 786 women with histologic evidence of PCOS in their late 20s there was no observed increase in CVD mortality at follow-up some 30 years later (76). The Dahlgren cohort was again evaluated at age 70 years. Of the 25 PCOS subjects evaluated and 68 controls there was evidence of elevated triglycerides and more hypertension in the cases but morbidity and mortality was the same across groups (33 cases and 95 controls) after controlling for BMI (77). FSH levels remained lower and free androgen index higher between cases and controls after menopause. The difference in waist hip ratio between cases and controls observed at younger ages were no longer evident in the older cohort and was likely due to weight gain among the controls. The authors also found a higher prevalence of hypothyroidism in controls (34%) compared to PCOS cases (8%) (78). The patients of the Pierpoint study were relatively young to be assessed for CVD morbidity and mortality and the Dalgren group small. These studies also evaluated events, we will evaluate women around the time of the menopause transition and examine the relationship between PCOS and menopause on CVD risk factors and SCA.

In 2008, Talbott et al evaluated the effect of menopause on coronary artery calcification in 128 cases and 166 controls participating in the third CHARM follow-up. PCOS continued to be associated with increased coronary calcification, however among PCOS women who reported surgical menopause their coronary artery calcification scores were significantly greater than controls undergoing surgical or natural menopause (79). Similar findings with regard to risk for progression of preclinical CVD as documented by intima media thickness (IMT) in women who experienced a more rapid menopause transition were published by Johnson et al in 2010. In their sample of 203 women with no prior diagnosis of CVD, those women who transitioned from a pre- to a post-menopausal state within the 3 year observation period exhibited an increased

rate of disease progression compared to those who were continuing to transition (11). Further, women undergoing bilateral oophorectomy before age 45 had a 1.44 times greater risk of dying from CVD compared to control population (80).

2.4 Aging and PCOS

There is a paucity of information on the menopause/perimenopause transition in PCOS. The knowledge gap related to reproductive aging in PCOS was recently identified as a priority research area by the ESHRE/ASRM (12). The final menstrual period (FMP) retrospectively defines menopause. Analogous to the interval immediately following menarche, the interval preceding the final menstrual period is associated with fluctuating hormone levels, changes in menstrual cycle number and duration, and intermittent ovulation. The effects as well as the other symptoms of menopause (hot flashes, sleep disturbances, change in mood) are related to ovarian aging and follicle demise as well as changes in the sensitivity of the hypothalamic-pituitary axis to sex steroid feedback (81). Just as the definition and diagnosis of PCOS was inconsistent, so too was the diagnosis of menopause. In 2001, the major professional societies for reproductive medicine, menopause, and NIH co-sponsored the Stages of Reproductive Aging Workshop (STRAW) to develop uniform criteria and nomenclature for staging reproductive aging in women. The STRAW menopause staging system is based largely on a woman's self-reported changes in menstrual interval and therefore not generalizable to those who have a history of irregular cycles, are extremely under or overweight, or smoke (82). By definition PCOS is associated with cycle irregularity and therefore the STRAW criteria would not be suitable for determining menopause transition in women with a history of the condition.

The timing of menopause is influenced by a number of modifiable and non-modifiable factors that include ethnicity and socieconomic status, weight, exercise, and smoking. Typically evidence of the perimenopause (i.e., menstrual irregularity) can be detected around age 45 with

menopause occurring about 6 years thereafter [For review see Gold E. 2011 *Obstet Gynecol Clin North Am* 38:425-440](83). The age and timing of the menopause transition is important because of the increase in long-term health risks to non-reproductive systems that are associated with early and rapid loss of estrogen (10, 11, 83).

Aging has been associated with normalization of menstrual cycle irregularities and androgen excess associated with PCOS. Elting and her colleagues published a retrospective study evaluating cycle patterns in 205 women previously diagnosed with PCOS (6). The average follow-up interval was approximately 12 years and participants were between 30 and 56 years of age. Sixty percent of the women participating reported a cycle interval of <6 weeks and this was inversely correlated with age ($p<0.001$). Logistic regression analyses accounting for BMI, weight loss, smoking, ethnicity, prior hormonal treatments, and prior pregnancy had no impact on the effect of aging on menstrual cycle frequency in PCOS. The team then prospectively evaluated 27 women with a prior diagnosis of PCOS who were between the ages of 36 and 50. Each underwent an FSH stimulation test to measure of ovarian reserve and received a transvaginal ultrasound scan to determine follicle number. Twenty women reported menstrual intervals of less than 6 weeks and seven women reported intervals greater than 6 weeks. Women with regular cycles were older (median age 40.9 yrs) and leaner (median BMI 24.3 kg/m^2) compared to women with irregular cycles (median age 38.8 years, $p<0.04$; median BMI 29.1 $kg.m^2$, $p<0.07$). Women with PCOS who reported regular cycles had significantly lower testosterone and androstenedione levels and higher FSH levels compared to those with irregular cycles. These women also had approximately half the number of ovarian follicles (median follicle number 8.5) compared to women with irregular cycles (median follicle number 18.0, $p<0.01$)(84). Another study of 472 women with a diagnosis of PCOS showed a statistically significant correlation between increasing age and decreasing cycle interval. Significant reductions in testosterone, androstenedione, LH, and follicle number in older (ages 30-42) compared to younger (ages 17-29) were also noted (85).

The effect of age on testosterone was previously evaluated in a subset of women participating in CHARM I. Winters et al documented a 50% reduction in total and free testosterone levels in PCOS cases 42-47 years of age compared to PCOS cases between 20 and 42 years of age (7). When they compared androgen levels between PCOS cases and normal reproductive controls by age quartile, statistically significant differences between groups at the youngest and oldest strata were observed but testosterone levels were comparable between cases and controls in the 42-47 year age quartile (7). The overall trend among PCOS cases was toward a reduction in androgen levels with increasing age however, testosterone levels remained higher in older PCOS cases compared to older controls suggesting continued ovarian responsiveness to LH or insulin stimulation.

Recently, two separate groups reported that both the adrenal gland (86) and the ovary (86, 87) contribute to postmenopausal hyperandrogenism in women with a prior PCOS diagnosis. Markopoulos et al evaluated 20 postmenopausal women with a past diagnosis of PCOS and 20 postmenopausal controls of similar age, years since menopause, BMI, and percent body fat. PCOS women registered greater waist circumference and waist to hip ratio as well as baseline elevations in androgen levels and free androgen index (FAI) and decreased sex hormone binding globulin (SHBG) levels compared to controls (86). When stimulated with adrenal corticotrophin releasing hormone (ACTH) postmenopausal PCOS and controls exhibited similar androgen responses. A 3-day dexamethasone suppression treatment yielded reductions in adrenal and ovarian hormones in both groups but PCOS exhibited less suppression of androstenedione, 17-OHP, and FAI compared to controls (86). Puurunen and colleagues evaluated 50 women in four groups; PCOS (n=11), controls (n=10), postmenopausal PCOS (n=18) and postmenopausal controls (n=11) before and after oral glucose tolerance testing and stimulation with human chorionic gonadotropin (hCG). They found persistent insulin resistance and high C-reactive protein in pre- and post-menopausal PCOS groups compared to pre- and post-menopausal controls. In addition, the postmenopausal PCOS group had

androstenedione responses to hCG stimulation that were nearly two fold greater than postmenopausal controls (1,000 ± 313.8 vs. 531.3 ± 40.3, p=0.035). The androstenedione response among postmenopausal PCOS and premenopausal controls was similar (87).

One longitudinal study exists demonstrating a change over time in reproductive indices and androgen levels among women with PCOS (88). A group of 254 women with PCOS defined by the Rotterdam criteria were evaluated at baseline and again approximately 2.6 (n=172) or 5.5 (n=84) years later. A reduction in serum testosterone levels was observed and a small proportion of women in the PCOS group (4.4% to 4.6%) reporting regular menses between the first and second visits (88). This investigation provided limited information on the menopause transition due to the young ages at baseline (24.9 to 30.9 years) and follow-up (35.5 years) (88).

Do older women with PCOS have more fertility? A large study from Norway evaluated birth rates in 500 women with PCOS and 500 control women who underwent IVF. These investigators found that older age was associated with a reduced number of oocytes retrieved and reduced live birth rates in control but not PCOS subjects (9) which suggests a longer window of fertility in women with PCOS.

Collectively, these data support the notion that women with PCOS have sustained ovarian function and conceivably have a different progression toward ovarian senescence compared to women with normal reproductive histories. Sustained ovarian activity may translate to longer duration of estradiol exposure and thus modify CVD risk factor progression among PCOS. Further, individual variability in ovarian activity across the reproductive window as evidenced by degree of cycle irregularity or degree of cycle control through use of oral contraceptives may influence CVD risk factors in this population. To the best of our knowledge this has not been examined in a large well characterized group of women with a history of PCOS. Ultimately, longitudinal studies are needed fully characterize reproductive aging and the menopause transition in PCOS and to explicate the relationship between menopause and chronic disease risk in this population.

2.5 Summary

PCOS affects a significant proportion of women, has a recognized metabolic component, and contributes to increased long-term health burden for the individual and society. Young women with PCOS present with increased CVD risk factors. Menopause furthers the risk for CVD among women. Limited data is available on the relationship between PCOS, menopause, and risk factors for cardiovascular disease. Our approach to address this knowledge gap evaluated data collected as part of the CHARM III study. This study included a group of well-characterized cases with a history of PCOS and a group of normal reproductive controls whose average age was between 47-49 years who submitted to a detailed reproductive history with hormonal assessments and evaluation of CVD risk factors and subclinical atherosclerosis. Understanding the relationship between menopause and CVD risk factors in women with PCOS is warranted so that appropriate management strategies can be developed and applied to facilitate healthy aging. The information garnered from this investigation may result in better strategies for management of PCOS through the menopause transition and ultimately improve health and quality of life for those with this disorder.

2.6 Tables Section 2.0

Table 1. Comparison of the National Institutes of Health (NIH), Androgen Excess PCOS society (AE-PCOS), and Rotterdam PCOS definitions for ten different phenotypic presentations

	Phenotypic Presentation	1	2	3	4	5	6	7	8	9	10
Feature	Hyperandrogenism	✓	✓	✓	✓			✓		✓	
	Hirsutism	✓	✓			✓	✓	✓	✓		
	Oligo/anovulation	✓	✓	✓	✓	✓	✓				✓
	Polycystic Ovaries	✓		✓		✓		✓	✓	✓	✓
Definition	NIH (1990)	+	+	+	+	+	+				
	AE-PCOS (2006)	+	+	+	+	+	+	+	+	+	
	Rotterdam (2003)	+	+	+	+	+	+	+	+	+	+

✓= feature present; + = meets definition; *Adapted with permission (19)*

Table 2. Major findings from Cardiovascular Health and Risk Measurement (CHARM) study (66-73,79)

Study	Year	Cases (n) Controls (n)	Age ± SD (yrs)	Primary Finding (s)
Talbott, et al	1995	206 206	35.9 ± 7.4 37.2 ± 7.8	• ↑ total cholesterol, LDL, triglycerides, and insulin and ↓ HDL in PCOS • Differences evident at earlier ages
Guzick, et al	1996	16 16	44.4 ± 3.6 43.9 ± 5.2	• ↑ IMT in PCOS vs. controls
Talbott, et al	1998	244 244	35.3 ± 7.4 36.7 ±7.7	• PCOS predictive of total cholesterol and LDL at younger rather than older ages
Loucks, et al	2000	63 56	35.0 ± 6.3 38.3 ± 6.7	• Polycystic ovaries did not alter CVD risk
Talbott, et al	2000	125 142	37.5 ± 6.2 39.0 ± 6.2	• Older PCOS cases had greater IMT that was not explained by age or BMI
Talbott, et al	2004a	61 85	47.9 ± 5.0 49.2 ± 5.4	• PCOS predicted CAC; effect mediated by insulin and HDL • PCOS 4Xs metabolic syndrome • Total testosterone risk factor for aortic calcification in PCOS and controls
Talbott et al	2004b	47 59	49.2 ± 4.0 49.5 ± 3.4	• No influence of CRP on IMT in PCOS • Obesity influenced CRP and IMT in PCOS • PCOS may have residual effect on IMT beyond insulin and fat mass
Talbott et al	2008	149 166	47.3 ± 5.6 49.4 ± 5.8	• Menopause risk factor of CAC • Surgical menopause more significant in PCOS

LDL-low density lipoprotein; HDL-high density lipoprotein; IMT- intima-media thickness; BMI-body mass index; CAC- coronary artery calcification; CRP-C reactive protein

3.0 METHODS

3.1 Subjects

The study population included women with PCOS and women with normal reproductive history evaluated in their middle to late reproductive years [mean (SD) age of cases 35.3 (7.4) and controls 36.7 (7.7) years] for cardiovascular risk factors between 1994 and 1995 (phase I) of the CHARM study (70). Participants were re-evaluated in 1997-1999 (CHARM phase II) for carotid intima-media thickness (IMT) and in 2000-2006 (CHARM phase III) for cardiovascular risk factors and coronary calcification, **Table 2**.

CHARM III included three visits that occurred during 2001-2003, 2003-2004, and 2003-2006. Visits 1 and 3 focused on measurement of coronary calcification and visit 2 focused on carotid IMT. The main hypotheses of this dissertation were evaluated from outcomes collected during CHARM III visit 1. A total of 152 cases and 169 controls between the ages of 35 and 67 years participated in CHARM III visit 1. CHARM III visit 3 occurred about 2.8 years later and included 121 cases and 154 controls that were evaluated previously at Visit 1, **Figure 1**. The exploratory analysis (Aim 3) included data from visits 1 and 3 in a subset of patients who were between the ages of 42-48 years at visit 1. This age range was selected as we were most interested in evaluating the outcomes (AMH, estradiol, and androgen levels) during the perimenopausal window when ovarian follicular activity wanes. The study was reviewed and approved by the Institutional Review Board of the University of Pittsburgh and all subjects provided written informed consent prior to the initiation of study procedures.

A detailed description of the approach to identify cases and controls in phase I has been published elsewhere (70). Briefly, PCOS cases were identified through retrospective review of medical records of women seen between 1972-1992 for infertility by Drs. David Archer and

David Guzick. Medical records resided within reproductive endocrinology and infertility division at Magee-Womens Hospital and were reviewed to determine eligibility for inclusion by Dr. Evelyn Talbott in 1993-1994. A presumptive diagnosis of PCOS was made based on history of chronic anovulation associated with clinical evidence of androgen excess (hirsutism) or if total testosterone exceeded 2 nmol/L or the LH/FSH ratio was > 2; criteria consistent with the 1991 NIH definition of PCOS (15). Neighborhood controls matched for age ± 5 years and race were recruited from 1992 voters' registration tapes for the greater Pittsburgh area and Cole's Cross Reference Directory of households (89). Controls reported regular menses and had no complaints of hirsutism or fertility problems. New subjects were added between phases I and III in an attempt to increase the heterogeneity of the study population. Newly added cases and controls met the same inclusion and exclusion criteria. Loss to follow-up of cases and controls made it impractical to maintain the matched design used in CHARM I in the later study phases.

3.2 Interventions

CHARM III visits 1 and 3 consisted of a detailed clinical interview that included demographic and behavioral information, current and past medical and surgical interventions, concurrent and previous medications, and family, reproductive, and menstrual histories. Statin use was not a specific question at CHARM III visit 1. To ascertain this information, participants' responses to questions about "other medications" were tabulated to approximate usage. A copy of the questionnaire used in the clinic interview appears in the **Appendix**. Anthropomorphic and blood pressure measurements were obtained as well as a fasting blood sample for evaluation of reproductive and androgen hormones and metabolic factors. Participants also underwent electron beam tomography (EBT) scanning for assessment of coronary artery calcification (CAC).

Current menstrual history included a description of the number and length of cycles in the previous twelve months and date of last menstrual period. If a participant reported no menstrual cycles in the previous year, they were asked when their cycle had stopped and the reason. Reproductive history included 1) prior reproductive surgery, type, and reason; 2) use of fertility medications; and 3) number of pregnancies and births. Participants were queried about current and past use of oral contraceptives (OCs), medroxyprogesterone (Provera), and hormone replacement therapy (HRT). Past use in months was recorded by decade beginning with the teenage years. The average number of menstrual cycles per year and average cycle length when not using hormonal contraception or pregnant was also recorded by decade.

Height (m) and weight (kg) measurements were recorded and utilized to calculate body mass index (BMI, kg/m^2). Waist to hip ratio (WHR) was determined from the average of two measurements taken at the minimum waist circumference and the maximum hip circumference (70). Systolic and diastolic blood pressures were determined from the average of two readings obtained using a random-zero sphygmomanometer. All participants were told to fast for at least twelve hours prior to the office visit. Venipuncture was performed and serum aliquots from blood samples were frozen at -80C for later analysis of reproductive hormones, androgens, lipids, insulin and glucose.

3.3 Laboratory Assessments

Laboratory determinations of insulin, glucose, and lipids were completed for all participants seen at visit 1 and visit 3. Luteinizing hormone (LH), follicle stimulating hormone (FSH), estradiol (E2), total testosterone (TT), and sex-hormone binding globulin (SHBG) levels were analyzed for all visit 1 participants. A subset of participants (38 cases and 40 controls) who completed visits 1 and 3 and were between the ages of 42 to 48 years at visit 1 were selected for

the exploratory analysis that included determination of anti-müllerian hormone (AMH) levels as well as LH, FSH, E2, TT and SHBG in serum collected at both visits 1 and 3.

3.3.1 Biochemical Determinations

Hormone assays were performed at Magee-Women's Research Institute and Emory University, in Atlanta, Georgia in the research laboratory of Dr. Sarah Berga. All analyses were run in duplicate and batched to reduce variability. LH and FSH were measured using a fluoroimmunometric assay (IFMA) (Delfia hLHSpec and FSH- Perkin Elmer, Fisher Scientific, USA). Interassay and intrassay coefficients of variation (CV) for these methods were less than 5%. AMH was measured by a highly sensitive enzyme linked immunoassay (ELISA-Beckman Coulter). This method has a sensitivity of 0.10 ng/mL and assay variation was between 4 and 8%.

Levels of E2 at visit 1 and and TT at visit 1 and visit 3 were measured by radioimmnoassay (RIA) of serum samples from using a solid phase RIA (Coat-A-Count, DPC, Los Angeles, CA) with between assay and within assay CVs of less than 10%. Estradiol levels were measured in visit 3 samples using an alternate fluroimmunometric method (Delfia-Perkin-Elmer, Fisher Scientific, USA) because of sample volume limitations. Five visit 1 samples were reanalyzed with the alternate method and the correlation was strong (r=0.999). Reported sensitivity of estradiol and total testosterone assays were between 10-14pg/mL and 4ng/dL respectively.

Serum SHBG levels were determined using an immunoradiometric assay (IRMA) (DSL, Webster, TX). This assay has a sensitivity of 3 nmol/L and required only 25µL of sample. CHARM III visit 3 specimens were evaluated using an alternate assay because the RIA was no longer available. Five specimens collected at visit 1 were reanalyzed using the replacement method and there was a strong correlation (r=0.910) between SHBG values using the original and replacement assays.

Calculations of the free androgen index (FAI) and free estradiol index (FEI) were made using the following formulas, FAI [(100 X TT ng/dL)/(28.84 X SHBG nmol/L)] and FEI [(100 X E2 pg/mL)/(272.11 X SHBG nmol/L)]. The ratio of testosterone to estradiol was then determined by calculating the ratio of FAI:FEI for each subject (13, 70, 90).

Lipid, insulin, glucose levels in the fasting blood samples were analyzed by the Heinz Nutrition Laboratory at the University of Pittsburgh Graduate School of Public Health according to standard methodology (70). Lipid profiles included total cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and triglycerides.

3.3.2 Homeostatic Model Assessment (HOMA)

Insulin resistance (IR) was estimated using fasting insulin and glucose levels obtained at CHARM III clinic visits 1 and 3. HOMA-IR was computed by dividing the product of insulin (μIU/mL) and glucose (mg/dL) by 405 (91).

3.3.3 Coronary Artery Calcification

Coronary artery imaging was performed by the Preventive Heart Care Center of the University of Pittsburgh Medical Center (UPMC) using an ultrafast CT scanner (Imatron C-150, San Francisco, CA). Detailed descriptions of the scanning procedure and calculation of CAC score have been published previously (74, 79). Briefly, technicians masked to the case or control status obtained thirty to forty 3mm sequential transverse images from root to apex in 100-msec exposures at 60% of the R-R interval of the cardiac cycle. Each participant was scanned once at each visit to minimize exposure to radiation. Images were analyzed with the AcuImage software using a base value region of interest (BVROI) approach. All pixels exceeding an area of $1mm^2$ and intensity of 130 Hounsfield units in each 3mm section were considered calcified. Calcification scores for each ROI were calculated using the formula (area of all significant pixels X grade of peak computerized tomography number); individual ROI scores were then summed to arrive at a total CAC score according to the method developed by Agatston et al (92).

3.4 Reproductive Variables Created in Present Investigation

3.4.1 Reproductive Designations

CHARM III included a detailed reproductive history and a single blood sample for reproductive hormones at visits 1 and 3. The investigation was not designed to evaluate the menopause transition. To address such a query in women with PCOS a study design incorporating more frequent sampling over a greater time span would be optimal. As reviewed earlier, the staging criteria for menopause (STRAW) are not reliable in predicting menopause in overweight women or those with history of irregular menses (82). Alternatively, a determination of whether the CHARM III participant was menopausal (surgical or natural), not menopausal, or unclear (currently using OCs or HRT) was made using a strategy adapted from the Women's Ischemia Syndrome Evaluation (WISE) study algorithm (93). A combination of menstrual cycle pattern in the prior twelve months, concurrent use of OCs or HRT, history of gynecologic surgery, age, and FSH and E2 levels were used to render a reproductive designation for participants at CHARM III visit 1.

Variability in FSH level is a hallmark of the transition from perimenopause to menopause and lab specific cut-offs based on a FSH value great than 2 SD above the mean level of FSH in a reproductively competent age group is recommended (82). FSH assay methods were unchanged between CHARM I and CHARM III. At CHARM I, the mean FSH levels in a subset of cases and controls ages 25-34 years who were not using hormones were 4.6 ± 1.6 and 5.9 ± 4.5 IU/L, respectively [from unpublished data from thesis dataset (69)]. Thus an FSH cut-off of 20 IU/L in the context of low E2 (< 50pg/mL) among women in CHARM III is conservative for those who reported that their cycles had stopped or who had undergone a hysterectomy.

Women who reported 5 or more months of amenorrhea prior to visit 1 and who had not had a hysterectomy were considered menopausal if they had an FSH level greater than 20 IU/L and estradiol level less than 50 pg/mL when not using hormone replacement. Similarly, women

who had previously undergone hysterectomy with one or both ovaries intact were considered definitely menopausal if they had an FSH≥20 IU/L and E2 ≤50pg/mL and were not currently using HRT or OCs. This group was designated as natural menopause.

Menopause status was also assigned to any woman with a history of a bilateral salpingo-oophorectomy (BSO) or chemotherapeutic treatment resulting in ovarian toxicity. Women with a spontaneous cessation of menses prior to age 40 and elevated FSH (≥20 IU/L) and low estradiol (≤50 pg/mL) were considered to have premature ovarian failure. These presentations were treated as a separate menopause group.

Women reporting a regular menstrual cycle or less than 5 months of amenorrhea were classified as not menopausal. Those women who previously underwent a hysterectomy and were not using hormones and had an FSH level <20IU/L were considered not menopausal.

The remaining subjects reporting current HRT use were classified as menopausal if they reported that their menses had stopped before starting HRT. Exogenous hormones mask underlying menstrual cycle pattern and endogenous hormone levels. Oral contraceptives have been shown to suppress LH and FSH levels in young women and young women with PCOS (39, 94). Likewise, Dupont et al showed that HRT suppressed LH and FSH in postmenopausal women however, levels remained higher than in premenopausal women (95). Further, women with a previous BSO and were placed on daily HRT for 12 months had mean (SD) FSH levels of 84.7 (31.1) IU/L (96). Similarly FSH was not suppressed to premenopausal levels in a Norwegian cohort of postmenopausal women using HRT (97). For these reasons and because our questionnaire captured timing of initiation of hormone replacement, a presumptive designation of reproductive status was made for women on HRT or OCs based on endogenous hormone levels and menstrual history. We approached the inclusion of these subjects conservatively in our analysis by conducting statistical evaluations with and without these subjects in our models.

A small number of cases and controls using HRT or OCs could not be classified because of equivocal hormone levels or use of HRT prior to spontaneous cessation of menses. These women were classified as unclear. One participant had amenorrhea for > 12 months and was not using HRT or OCs, exhibited hormonal pattern consistent with hypothalamic hypogonadism. This participant was currently using gabapentin which has been shown to be effective for managing hot flashes (98). Studies in male rats have shown that gabapentin suppresses FSH and testosterone levels and reduces fertility (99). This participant was also classified as unclear.

3.4.2 Duration of Menopause

The duration of menopause was calculated from the age at which a participant reported their period had stopped to age at study visit. This measure could not be determined for women who underwent a hysterectomy without bilateral oophorectomy because the study was not designed to include a sampling frequency to assess biochemical markers of ovarian function in the absence of menses. Based on recent guidelines from the follow-up to STRAW (STRAW +10) where rationale for categorizing the post menopause by duration in consideration of continued physiologic changes including rising to FSH and diminishing estradiol levels and symptoms of estrogen deficiency such as vaginal and urogenital complaints (100) a grouping variable based on duration was created for menopausal women in CHARM. Categories were based on the recommendations from the follow-up report from the Stages of Reproductive Aging Workshop, ≤2 years, 2-6 years, and >6 years since final menstrual period(100). When possible, suspected timing of menopause was determined for women who had previously undergone hysterectomy based on timing between surgery and age at CHARM III visit 1.

3.4.3 Lifetime Cycle Irregularity

Women with PCOS report varying degrees of menstrual irregularity that could overtime translate to differential exposures to sex steroids and thus influence CVD risk factors or

subclinical marker of CDV. Women participating in CHARM III visit 1 were asked to estimate the number and duration of cycles they had per year during each decade from the teens through current decade when not using hormonal contraception or pregnant. They were also queried about hormone use and birth of children across each decade. This information was used to devise a metric for overall menstrual irregularity. The steps to quantify lifetime irregularity are outline below.

1) *Total reproductive years*
    a. The difference between the participant's current age or age at menopause and age at first menses.
    b. This number was then subdivided by decade to determine the number of reproductive years contributed during the teens, 20s, 30s, and 40s.
2) *Number of natural cycles per decade*
    a. This figure was determined by multiplying the number of periods per year when not pregnant or on oral contraceptives by the number of reproductive years contributed in the decade.
3) *Cumulative natural cycle number*
    a. The total number of natural cycles was calculated as the sum of cycles contributed in each decade.
4) *Live birth and oral contraceptive adjustment*
    a. Pregnancy is associated with a significant increase in estradiol 2.5 ng/mL in the first trimester to 15 ng/mL by the time of delivery (101), thus any pregnancy resulting in a live birth was assigned 10 cycles and added to the cumulative cycle number. Multiple gestations were treated as one pregnancy.
    b. OCs effectively suppress androgen levels (39, 94) in women with PCOS and do not appear to have a negative effect on metabolic parameters (94, 102). Thus the duration of OC use reported was added to the cumulative cycle number.

5) Lifetime irregularity

a. Overall lifetime irregularity reflects the combined contributions of natural cycles, pregnancy, and OC use.
b. To account for differences in ages, total lifetime irregularity was divided by the total reproductive years contributed.

The approach for calculating lifetime cycle irregularity is outlined in **Table 3** and includes an examples based on an actual case.

3.5 Statistical Analyses

3.5.1 Specific Aim 1

Specific Aim 1: examine the association between menopause and CVD risk factors and CAC in women with a history of PCOS and controls adjusting for age, body mass index, as well as other known predictors of CVD risk factors.

Hypothesis 1: menopause will further exacerbate risk factors for CVD and subclinical atherosclerosis (SCA) in older women with a history of PCOS compared to controls.

Descriptive analyses were completed and univariate assessments of differences at CHARM III visit 1 between combined cases and controls as well as cases and controls in four age and four reproductive strata were performed. Mann-Whitney U-test was used to evaluate differences in continuous variables between cases and controls within each strata. Kruskal-Wallis was then used to examine differences in outcome measures within cases or controls across strata. The χ^2 test was used to evaluate differences in categorical measures.

To evaluate potential interaction effects between PCOS status and reproductive status on CVD risk factors a two-way analysis of variance (ANOVA) was conducted. The effect of control or case (PCOS) status on the risk factor was examined across three reproductive strata (non-menopausal, natural menopause, and surgical menopause). A p value of <0.05 was considered significant.

Multiple linear regression models were constructed to examine the effect of case or control status on cardiovascular risk factors adjusting for type of menopause, age, and body mass index. Models were created by evaluating the effect of PCOS status on the risk factor of interest and then introducing each covariate into the model. To examine the relationship between natural or surgical menopause and PCOS on the risk factor, separate dummy variables were created for natural and surgical menopause and the interaction between these variables and PCOS. Non-normally distributed variables were log transformed before being entered into the models.

CAC was highly skewed; most participants had values with Agatson Scores at or very near zero. A dichotomous variable for CAC (<10 or ≥ 10 Agaston Score) was created and logistic regression used to evaluate predictors of CAC among cases and controls. An interaction term for PCOS and menopause was included in these models.

Based on previous experience with this cohort it was known that age differed between cases and controls. A subset of sequentially age ± one year and ethnicity matched cases and controls was created from the CHARM III visit 1 participant cohort. To avoid potential confounding effects of hormone use and premature menopause, cases and controls currently using OCs or HRT preparations or who had undergone pelvic surgery and experienced a cessation of menses or who experienced premature or medical menopause were excluded. The matched subset was similarly evaluated to determine if there was a difference in the occurrence of natural menopause between cases and controls.

3.5.2 Specific Aim 2

Specific Aim 2: examine the association between degree of menstrual irregularity, determined by number of cycles reported across earlier reproductive years, and CVD risk factors and CAC among women with PCOS who were evaluated during later reproductive to menopausal years.

Hypothesis 2: greater menstrual irregularity across time will be associated with a more adverse CVD risk profile and SCA documented by CAC in women with a history of PCOS.

Between and within subject comparisons for the change over time in the number of cycles during each decade were evaluated using a repeated measures analysis of variance. Cases were stratified based on degree of cycle irregularity (< 9 cycles/year, 9-11 cycles/year, and more than 11 cycles/year) and the Kruskal-Wallis test was used to evaluate univariate differences between PCOS cases by degree of cycle irregularity. Chi square was used to evaluate differences in categorical outcomes. Logistic regression models were constructed to evaluate the effect of cycle irregularity adjusting for other predictors on the presence of CAC among cases.

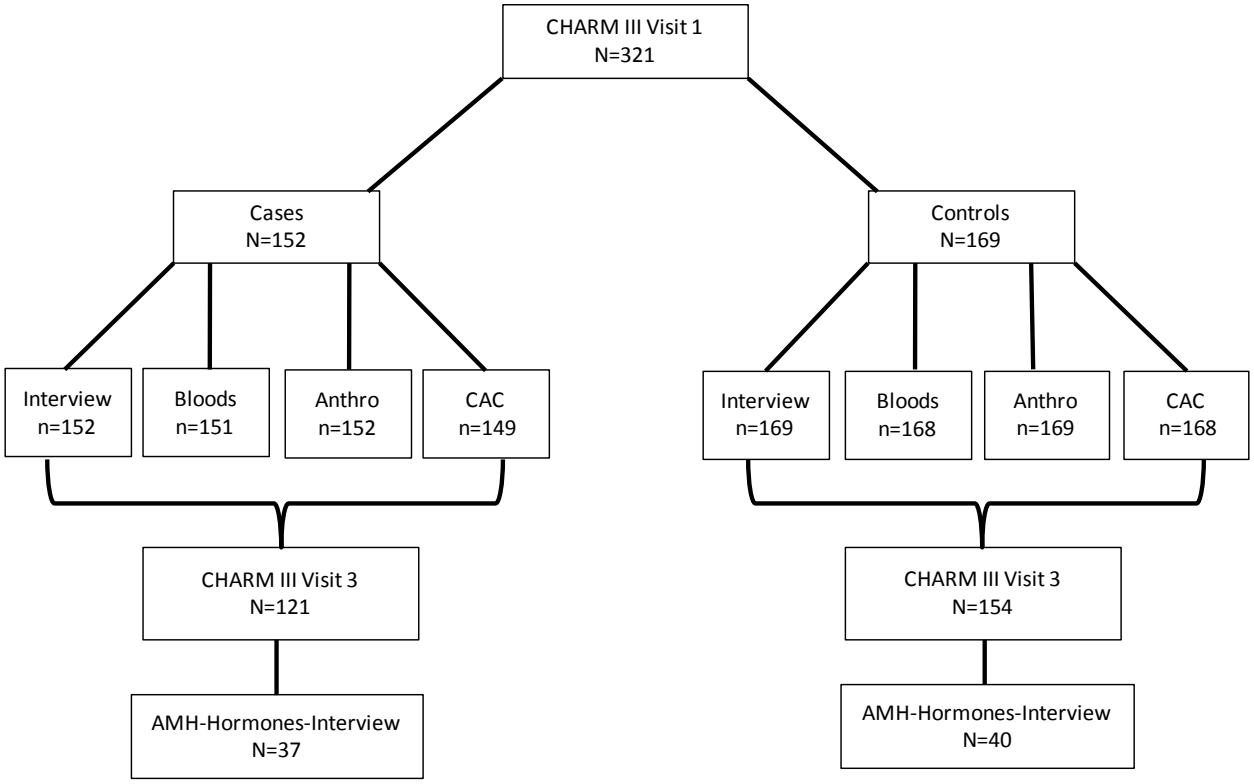
3.5.3 Specific Aim 3

Specific Aim 3: examine the relationship between ovarian reserve, documented by anti-müllerian hormone (AMH) level and relative testosterone to estradiol ratio, documented by the ratio of free androgen index to free estradiol index (FAI/FEI) in late reproductive age women with PCOS compared to controls.

Hypothesis 3: decreases in AMH level will be associated with an increased FAI/FEI ratio in both PCOS and controls. PCOS will have a smaller change in FAI/FEI that correlates with a higher AMH level.

Cases and controls were sequentially matched based on age at CHARM III visit 1. A repeated measures analysis of variance was used to evaluate potential differences in the parameters of age and BMI from visit 1 to visit 3 within and between subjects. Differences in the proportion of women in each group reporting cycles and using hormones between visit 1 and visit 3 were evaluated using McNemar's test. Percent change relative to the baseline ([(V3 – V1)/V1]*100) was computed for each outcome variable. Comparisons were conducted using Mann Whitney test. Spearman correlations were used to examine potential relationships between change in AMH and FAI/FEI between visits within cases and controls.

3.6 Figures and Tables for Section 3.0



Anthro: Anthropomorphic Measurements
CAC: Coronary Artery Calcification
AMH: Antimüllerian Hormone

Figure 1. Phases of the Cardiovascular Health and Risk Measurement (CHARM) III Study

Table 3. Lifetime cycle irregularity calculations

Step		Calculation	Example 1-Case				Example 2-control			
			Current Age: 52, Menarche: 12, Cycling: Yes				Current Age: 52, Menarche: 12, Cycling: Yes			
			OCs: 60 months during 30s				OCs: 60 months during 30s			
			Live Births: 3 total (2 during 20s; 1 during 30s)				Live Births: 3 total (2 during 20s; 1 during 30s)			
1)	Reproductive Years	Age at visit (or menopause) – Age at menarche	52 - 12 = 40yrs				52-12 = 40 yrs			
2)	Number of Natural Cycles/ decade	Periods/year (PPY) X number reproductive years/ decade	Decade	Repro yrs	PPY	Natural Cycles	Decade	Repro yrs	PPY	Natural Cycles
			Teens	8	6	48	Teens	8	10	80
			20s	8.3	5	41.5	20s	8.3	12	99.6
			30s	4.2	5	20.8	30s	4.2	12	50
			40s	10	10	100	40s	10	12	120
3)	Cumulative Natural Cycles	Natural cycles teens + 20s + 30s + 40s	210.3 cycles				349.6 cycles			
4)	Live births	Pregnancies resulting in live births X 10 months	30 cycles				30 cycles			
5)	Oral contraceptives	Months of OC Use teens + 20s + 30s + 40s	60 cycles				60 cycles			
6)	Adjusted lifetime cycle number	Cumulative natural cycles + Pregnancy Months + OC months	300.3 cycles				439.6 cycles			
7)	Adjusted lifetime cycles/ reproductive year	Lifetime irregularity/total reproductive years	7.5 cycles/yr				11 cycles/yr			

4.0 RESULTS

4.1 Aim 1

CHARM III, visit 1 included a total of 321 subjects (152 PCOS cases and 169 controls) age 35 years or greater. Subjects were predominantly Caucasian and educated beyond the twelfth grade, **Table 4a**. Cases were statistically significantly younger (mean ± SD) than controls (47.2 ± 5.6 vs. 49.5 ± 5.9, Mann-Whitney U-test p<0.001) (**Figure 2**). Because the age distribution of cases and controls differed, four age strata were created: 1) less than age 45 years, 2) ages 45-49 years, 3) ages 50-54 years, and 4) age 55 years or greater. Subject characteristics and outcomes presented in **Tables 4a** (baseline characteristics), **4b** (metabolic outcomes), and **4c** (reproductive parameters) include all cases and controls and subjects stratified by age group. Cases were more obese with greater central adiposity (waist to hip ratio) compared to controls, **Table 4a**. When stratified by age group, cases younger than age 55 years showed significantly higher BMI and waist to hip ratio compared to controls.

Comparable numbers of women reported use of statins in both the case and control groups, **Table 4a**. More cases were using diabetes medications 10.3% compared to controls 3.9%, $\chi 2$ p=0.035. Due to small numbers this difference was no longer significant when stratified by age. Cases in the youngest age strata (<45 years) had somewhat higher use 21.3% of anti-hypertensive medications compared to controls in the same age strata 6.3%, $\chi 2$ p=0.068. This difference was no longer apparent in older age strata. There were significant numbers of missing observations for use of antihypertensive (n=112 cases; 108 controls), diabetes (metformin) (n=107 cases; 101 controls), and insulin (n=101 cases; 101 controls) medications in the CHARM III, visit 1 dataset.

As shown in **Table 4b**, cases had lower total HDL and higher triglyceride and insulin levels relative to controls. Type 2 diabetes, as defined by self-reported history or fasting glucose

≥ 126 mg/dL, was three times more prevalent in cases compared to controls (12.5% vs. 3.6%, $p<0.01$) and insulin resistance (HOMA-IR) was also significantly greater in PCOS compared to controls ($p<0.001$). Differences in risk factors for cardiovascular disease and diabetes persisted between the cases and controls of the intermediate age groups (45-54 years). Diabetes remained significantly higher among the cases 45 years and older. When stratified by age cases exhibited a greater degree of insulin resistance at ages less than 55 years. Cases had greater coronary artery calcification scores in all age groups compared to controls and the difference was most evident between cases and controls in the two older age strata, **Table 4b**.

While the average age of cases and controls in the 50-54 year age group was comparable, a greater proportion of controls reported natural menopause 42.2% compared to 22.2% of cases ($p<0.05$), **Table 4a**. However, in women over 55 years the reverse was noted. At CHARM III visit 1 cases generally fit the typical hormonal profile observed in earlier CHARM studies; higher LH:FSH ratio and total testosterone level and lower SHBG level compared to controls, **Table 4c**. When stratified by age older cases consistently exhibited significantly lower LH and FSH levels compared to control counterparts. TT was significantly higher in cases compared to controls in the youngest age strata and comparable between cases and controls at ages greater than 45 years. SHBG was lower in cases of the two youngest strata but comparable in women 50 years and older. Estradiol did not differ between cases and control at any age. The free androgen index was statistically significantly higher in cases compared to controls in all strata. The free estradiol index was higher in cases compared to controls less than 50 years of age and decreased with age and was similar levels between cases and controls >55 years. FAI to FEI ratio differed between combined cases and controls. However, the difference was no longer significant when stratified by age. The FAI:FEI ratio rose across age strata for cases and controls and was comparable for cases and controls >55 years, however this trend did not reach the level of statistical significance.

A significant positive correlation between HOMA-IR and BMI was observed in both cases and controls (Spearman r^2=0.450, p<0.001). SHBG level was significantly and inversely correlated with HOMA-IR (Spearman r^2=-0.279, p<0.001) and BMI (Spearman r^2=-0.434, p<0.001). No statistically significant correlation between FSH level and BMI (Spearman r^2=-0.034, p=0.623) or insulin resistance (Spearman r^2=-0.008, p=0.924) was observed among cases.

At CHARM III visit 1, forty-five (30%) cases and seventy-nine (47%) controls reported that their menstrual cycles had stopped. Twenty-two (49%) cases and thirty-four (43%) controls reported either hysterectomy with or without bilateral oophorectomy as the reason for their periods stopping. **Figure 3** depicts the various reasons for cessation of menses in cases and controls. Twenty-two cases (14.5%) and thirty-four controls (20.4%) reported having a hysterectomy with or without a BSO or unspecified uterine/ovarian surgery associated with the cessation of their menstrual cycles. There was no difference in the proportion of participants having hysterectomy between cases and controls, $\chi 2$ p=0.168. Participants at CHARM III visit 1 were classified as not menopausal (96 cases and 79 controls), menopausal (19 cases and 38 controls), surgical menopause (11 cases and 17 controls), or unclear (25 cases and 33 controls), **Figure 4**.

Cases and controls were similar with regard to age at first menses, number of pregnancies, and OC use but cases were more likely to have used fertility medications and had fewer live births compared to controls, **Table 5**. The average duration of surgical menopause was significantly longer than natural menopause (108.4 (98.4) months vs. 60.2 (50.7) months, Mann-Whitney p=0.037) in all participants. The mean (SD) duration of natural menopause was similar for cases 61.5 (58.5) months and controls 59.7 (48.3) (Mann-Whitney p=0.945), not shown in Table 5. Likewise the duration of surgical menopause did not differ between cases and controls (134.0 (148.2) months vs. 97.1 (70.6) months, Mann-Whitney p = 0.894). Menopause duration could not be computed for women who stopped menstruating due to hysterectomy

without bilateral oophorectomy or who did not provide information on when their cycle stopped.

Within the unclear group of 25 cases and 34 controls, a presumptive determination of menopause or not was made for 17 cases and 25 controls. We conducted our analysis with and without these subjects and our findings were statistically comparable. The results presented in **Tables 6-8** include these subjects**. Tables 6a and 6b**, provide an overview of subject characteristics stratified by menopause status. Menopausal cases and controls were about ten years older than cases and controls who were not menopausal. Age was comparable within the non-menopausal and natural menopausal strata, but cases were who had surgical menopause were slightly younger than controls with surgical menopause but the difference did not reach statistical significance. Ages differed significantly across reproductive groups (ANOVA $p<0.001$). Post hoc testing with a Bonferroni correction identified that women who were classified as not menopausal were significantly younger than the natural, surgical and unknown groups ($p<0.001$). Women with surgical menopause were significantly younger than those with natural menopause ($p<0.001$) and comparable to the unknown group.

Cases who were not menopausal or who had natural menopause were more obese and had increased central adiposity compared to controls. Cases designated as not menopausal had significantly higher SBP and somewhat higher DBP compared to control counterparts. In addition, cases who were not menopausal had higher triglyceride and lower HDL levels compared to controls. Menopausal cases and controls had comparable blood pressures, HDL, and LDL levels. Triglyceride levels did not differ significantly but cases reporting natural menopause had a median triglyceride levels 10 points higher than control counterparts. Cases who were not menopausal as well as cases in the natural and surgical menopause groups had increased insulin resistance and insulin levels compared to controls. Most striking was that 25.0% of cases with natural menopausal had type 2 diabetes versus 1.9% of controls ($p<0.01$). Evidence of CAC was also significantly higher in both non-menopausal and menopausal cases

compared to controls. Significantly more cases in both the menopausal and not menopausal groups exhibited CAC exceeding 10 Agatston units compared to controls, **Table 6a**.

The ratio of LH to FSH, total testosterone level, FAI, FEI, and FAI:FEI were higher and SHBG level was lower in cases who were not menopausal compared to controls who were not menopausal (**Table 6b**). LH and FSH levels were significantly lower in menopausal cases compared to controls. Estradiol, and testosterone levels were comparable but FAI and FEI were significantly greater in cases compared to controls that had natural menopause. This can be attributed to the significantly lower levels of SHBG observed the cases with natural menopause. The median FAI was higher and median SHBG level lower in cases with surgical menopause compared to controls with surgical menopause but the differences did not reach statistical significance.

A factorial ANOVA showed no significant effect of group ($p=0.251$) but a significant effect of reproductive status ($p=0.028$), with no interaction ($p=0.765$) on SBP. Neither group ($p=0.184$) nor reproductive status ($p=0.719$) had any effect on HDL cholesterol level, nor was there a significant interaction ($p=0.685$). Triglycerides were associated with significant effects for both group ($p=0.021$) and reproductive status ($p=0.009$) and were non-significant for interaction ($p=0.844$). **Figure 5** shows the relationships between group (control or PCOS case) and reproductive status (not menopausal, natural menopause, or surgical menopause) on systolic blood pressure, HDL cholesterol, and triglycerides. Triglyceride levels were log transformed for the analysis.

Parameters from multiple linear regression models are shown in **Table 7** and after adjusting for age and BMI, PCOS status was not predictive of SBP or HDL but was a predictor of triglyceride level. Neither surgical nor natural menopause predicted SBP or HDL added any further impact to the risk of increased SBP or decreased HDL when included with age, BMI, and PCOS. However, surgical menopause was significantly associated with increased triglyceride levels. The effect of hormone used however negated the effect of surgical menopause on

triglycerides. Hormone use was also positively associated with HDL when modeled with age, BMI, PCOS status and menopause. Older age and increased BMI were predictive of SBP. BMI was most predictive of lower HDL level and higher triglyceride level. Because SHBG, HOMA-IR and BMI are inter-related and highly correlated with PCOS status these variables were not used as separate predictors in the regression models. There was no interaction effect of PCOS and any type of menopause in the models tested.

Logistic regression was performed to evaluate predictors of CAC score of ten or greater. As shown in **Table 8**, PCOS remained a significant predictor of increased CAC score (2.385, 95% CI 1.195, 4.762, p = 0.014) when menopause type, BMI, and age were included in the model. Natural menopause was also predictive of a CAC greater than 10 (4.579, 95% CI 1.599, 13.060, p=0.005).

The matched subset included 83 cases and controls. Eleven (13%) of the pairs were African American. As a group, cases were heavier with greater central adiposity compared to controls, **Table 9**. The proportion of cases and controls who had experienced natural menopause was not statistically different (18.1% vs. 27.7%, $\chi 2$ p = 0.192) and the duration of menopause was similar between cases (61.5 ± 58.5 months) and controls (60.5 ± 50.4 months), Mann-Whitney p=0.943. Cases and controls in this age matched subset were comparable in age and in proportion and duration of menopause. Other outcomes between the two groups were comparable to those observed in the larger cohort, **Tables 10 and 11**.

4.2 Aim 2

We next evaluated whether degree of menstrual irregularity at younger ages was predictive of CVD risk factors among older women with PCOS. The average number of natural cycles per year at each decade was significantly lower for cases compared to controls RM

ANOVA p=0.042, but increased similarly across each decade from the 20s RM ANOVA p<0.001, **Figure 6**. The median (IQR) of natural cycles at each decade is shown in **Table 12.**

Cases with the fewest cycles over time had higher total testosterone, FAI, and FAI to FEI ratio compared to cases with more frequent cycles, **Table 13**. There were no differences in CVD risk factors, prevalence of diabetes or % CAC between cycle groups. Subjects with the greatest degree of irregularity had higher total average CAC scores compared to those cases with less irregularity, however there was not a statistically significant difference between groups.

When evaluated in a logistic regression model, the greater cycle irregularity did not predict greater CAC when adjusting for age and insulin resistance, **Table 14**.

4.3 Aim 3

A total of 28 cases and 28 sequentially age ± 1 year matched controls were included in Aim 3. Subjects were between the ages of 42 and 48 years at CHARM III visit 1, not menopausal, and were not currently using OCs or HRT. As depicted in **Table 15**, the percent change in age from visit 1 to visit 3 was comparable between cases (6.8%) and controls (6.3%) (Mann-Whitney p=0.43). A repeated measures ANOVA documented that cases and controls were comparable in age (p=0.29) at each visit and increased similarly between visit 1 and visit 3 (p<0.001). Further, cases and controls did not experience significant differences in percent weight loss or weight gain between visit 1 and visit 3 (Mann-Whitney p=0.93). Further, BMI was comparable between cases and controls (Repeated Measures ANOVA p=0.51) and across each visit (Repeated Measures ANOVA p=0.88).

The proportion of cases reporting cycles did not change significantly between V1(96.4%) and V3 (82.1%) (McNemar p=0.125). Similarly, there was not a statistically significant change in among controls reporting cycles from visit 1 (96.4%) to visit 3 (85.7%) (McNemar p=0.250).

Hormone profiles including AMH levels for this subset women appear in **Table 15**. There was a statistically significant difference between cases and controls for change in AMH level across the visits. However, AMH level was near the limit of detectability for the assay.

A Spearman's correlation was used to determine associations between biochemical markers evaluated at visit 1 and changes in biochemical markers evaluated at visit 1 and visit 3 in cases and controls. An inverse association between age and AMH level was observed in cases (r_s=-0.442, p=0.018) and controls (r_2=-0.483, p=0.009) at visit 1. Similarly, AMH and FSH were inversely associated at the initial visit in controls (r_s=-0.426, p=0.024) but not cases (r_s=-0.248, p=0.203). There was no association between change in AMH between visits and change in FSH, androgens, estradiol or SHGB level in either cases or controls. However, change in BMI among cases was directly correlated with change in insulin resistance (r_s=0.424, p=0.031) and inversely correlated with change in SHBG level (r_s=-0.485, p=0.010). Further, BMI change was positively associated with change in FAI (r_s=0.410, p=0.034) and FEI (r_s=0.407, p=0.035). An inverse association between change in BMI and change in HDL was also observed (r_s=-0.395, p=0.037).

5.0 CONCLUSIONS

Our overall objective was to examine the relationship between reproductive status and menstrual irregularity on CVD risk factors and sub-clinical atherosclerosis in women with a history of PCOS and normal reproductive control subjects. The study population was initially described in 1993-1994 when the cases and controls were on average in their mid- to late thirties. At CHARM III, about ten years later, those women enrolled as PCOS cases exhibited a presentation consistent with the reproductive, endocrine and metabolic profile generally ascribed to the condition i.e., irregular periods, high LH to FSH ratio, increased androgens, insulin resistance, and CVD risk factors. When stratified by age or menopause, only younger cases (ages 45-49) were observed to have a more consistently adverse CVD risk profile compared to controls as evidenced by greater obesity and central adiposity, higher SBP and triglyceride levels, and lower HDL. Differences in BMI and WHR between cases and controls persisted in cases with natural menopause but not in cases with surgical menopause. Blood pressure and lipid levels were comparable between menopausal cases and controls.

Multiple linear regression models showed that neither history of PCOS or menopause status predicted an increase in SBP or decrease in HDL when adjusting for age or BMI. This suggests that the independent contribution of PCOS to CVD risk profile observed between younger cases and controls in CHARM diminishes with increasing age.

Overall 12.5% of cases compared to 3.6% of controls had type 2 diabetes. Menopausal cases exhibited significantly greater type 2 diabetes than younger or control counter parts. According to the CDC, 10.4% of white females between the ages of 45-64 have diabetes whereas 18.9% of black females in this age range have diabetes. These percentages increase to 16.9% and 31.2% respectively in women ages 65-74 (103). At CHARM III visit 1, 25.0% (n=6) cases with

natural menopause had diabetes. Another 28.6% (n=4) cases who reported surgical menopause were also diabetic. Nine of these 10 women were white and all were younger than 65 years. Thus, in CHARM III we observed diabetes prevalence rates more than twice that of the general female population. The rates of diabetes in white menopausal women with a history of PCOS were on the order of those observed among older black women. These findings coexist with a decrease in SHBG level in cases relative to controls. A recent review by Le et al, summarized available data including genetic evidence on the associations between low SHBG and risk for type 2 diabetes and suggests that SHBG is a biomarker for elevated insulin and glucose (104). In the CHARM III visit 1 data set HOMA-IR and BMI were significantly positively correlated in cases and controls (Spearman r^2=0.450, p<0.001). SHBG level was significantly and inversely correlated with HOMA-IR (Spearman r^2=-0.279, p<0.001) and BMI (Spearman r^2=-0.434, p<0.001).

We also observed significantly lower levels of gonadotropins (FSH) in menopausal PCOS compared to controls. There is evidence that at least 25% of men with type 2 diabetes have hypothalamic hypogonadism which is characterized by very low LH and FSH and accompanying low levels of free testosterone (105). Further, obesity in women has been associated with lower levels (106). No statistically significant correlation between FSH level and BMI (Spearman r^2=-0.034, p=0.623) or insulin resistance (Spearman r^2=-0.008, p=0.924) was observed among cases and this is likely due to the wide variation in FSH across the two groups. However, among women with natural menopause FSH levels were significantly inversely correlated with BMI (r_s=-0.527, p=0.001) and insulin resistance (r_s=-0.535, p=0.001). When evaluated separately, this relationship held within controls and cases who had undergone natural menopause.

Compared to controls, cases who had natural menopause recorded significantly higher estradiol levels that could also contribute to FSH suppression. However, there was not a statistically significant correlation between FSH and estradiol.

While CVD risk factors did not appear to worsen in menopausal women with a history of PCOS after adjusting for age, BMI, and hormone use, there was evidence of that subclinical atherosclerosis as measured by CAC was influenced by PCOS status and menopause independent of age and BMI. CAC was greater in PCOS compared to controls and this increase in subclinical atherosclerosis was most evident in the middle and older age strata. The odds of having a CAC score greater than ten were about 2 times higher in women with a history of PCOS compared to controls and 4.5 times higher following natural menopause when compared to non-menopausal controls. This relationship was preserved even after including age and BMI and is consistent with earlier reports by Talbott et al (79). However, the relationship between CAC and surgical menopause was not observed in PCOS cases in this evaluation and that may have to do with how the reproductive status was defined. In particular, women using HRT were classified as unclear and these women as a group had healthier profiles and lower CAC (**Table 6a**) and may have been included in the 2008 report as menopausal. Additionally, women with hysterectomy and intact ovaries were not classified as surgical menopause in this current analysis but may have been part of the surgical designation of the prior analysis. An evaluation of discordant group assignments will be necessary to clarify this discrepancy.

We found no differences in CVD risk factors or CAC in an older group of women with a history of PCOS stratified by degree of lifetime menstrual irregularity. More irregular (less frequent) cycles were associated with statistically significantly higher total and free androgen levels and FAI to FEI ratio. Logistic regression did not reveal that degree of cycle irregularity in cases across time was associated with an increased odds of CAC greater than 10 when age and insulin resistance were included in the model. The association between CAC and insulin resistance is not surprising; the observation that 25-29% of postmenopausal cases have type 2 diabetes supports the importance of diabetes prevention measures being introduced early for women with a prior history of PCOS approaching menopause.

The exploratory aim identified a statistically significant change in AMH level between the two CHARM III visits among PCOS cases. AMH levels in PCOS cases at visit 3 were comparable to AMH level of controls at visit 1. Caution is needed when evaluating any significance of this result due to the younger age of the PCOS group. Also, levels of androgen hormones and estradiol were near the assays' detect limits. Other metrics examined did not differ between cases and controls and that could be due to a small sample size. However, we did find significant correlations between changes in BMI and CVD risk factors and insulin resistance in this small group of women. These data combined with the findings of greater type 2 diabetes in PCOS following menopause suggest that perimenopausal weight gain could trigger transition from insulin resistance to diabetes and further progression of CVD RF and subclinical atherosclerosis. However, in the cross-sectional evaluation of the large group at CHARM III visit 1, BMI was comparable between non-menopausal and menopausal cases (**Table 6a**). Given the small sample size and significance of the findings a re-evaluation and expansion of the sample size would be a compelling effort. The limitation for this aim was related to having only a small number of subjects with specimens available for measurement of reproductive hormones. However, metabolic indicators, lipids, medical and reproductive histories were collected on all subjects and could be expanded to strengthen the analysis.

Many women in both the case and control group reported that they used OCs or medroxyprogesterone acetate (MPA/Provera) for contraception or menstrual cycle regulation. It is conceivable that the androgen suppressive effects of oral contraceptives may have had a positive effect on CVD risk profiles in older PCOS cases. There is evidence that OCs do not worsen CVD risk factors. The use of combined OCs in young eumenorrheic controls (n=10) and PCOS cases (n=9) was associated with decreased insulin sensitivity in both groups and an increase in triglyceride levels of only the controls(94). Cholesterol and HDL were comparable between controls and PCOS at baseline and after 3 months of continuous OC administration (94). Further, a recent meta-analysis examined the literature for associations between combined

OCs and metabolic indicators in women with PCOS from 35 different studies. Overall OCs increased HDLc and triglyceride levels significantly and there were no significant changes in LDLc, total cholesterol, insulin or glucose (102).

5.1 Study Limitations

Certainly the age difference between cases and controls presented a challenge particularly when attempting to evaluate the contribution of an age related reproductive processes to cardiovascular risk. We evaluated this in a small subset of age matched cases and controls (83 per group) and found no differences in the proportion of subject who were menopausal. A much larger sample size and a different study design would be needed to fully compare the menopause transition between cases and controls. Also, the cases and controls in this study were largely white and well educated. In addition, cases were identified from an infertility practice and as such may have had a different course due to earlier interventions. For these reasons, our finding may not be generalizable to a larger more diverse population.

There are a few studies where lifetime estrogen exposure has been attempted. Each touches on the challenges of recall bias and reporting of cycle history and hormone use. CHARM is no different. Lifetime cycle irregularity was shown to be associated with higher androgen levels at CHARM III visit 1. The study does not permit evaluation of hormone/androgen exposure over time. These measures are also subject to recall bias and may not be the most accurate reflection of estrogen exposure across time particularly in women with irregular menses. Moreover, menstrual bleeding is not verification of ovulation and in this study could be an over estimate of what could be a normal estrogen exposure. An exercise to examine consistency of a subjects' recall across the three CHARM phases would be to evaluate in a subset of cases and controls individual responses to questions pertaining to cycle frequency and cycle duration that were provided at each clinical interview during phases I, II, and III for

the same decades. Other challenges to deriving this number is that information on breastfeeding and lactational amenorrhea was not obtained nor was information on specific type of HRT consistently obtained.

6.0 FUTURE DIRECTIONS

While the definition and classification of PCOS continues to be debated, we know that the clinical presentation of menstrual irregularity in the context of ovarian hyperandrogenism and metabolic syndrome places a woman at increased risk for long term negative health consequences. We were not able to determine the trajectory of ovarian senescence in our study nor do the findings presented here suggest that menopause independent of age further amplifies cardiovascular disease risk factors in women with a history of PCOS. However, our finding do support the notion that when type 2 diabetes manifests in older women with a history of PCOS there is increased evidence of early atherosclerosis. In this study the participants were relatively young and had generally managed their PCOS symptoms for decades beginning with their presentation for infertility evaluation. The majority had a primary physician, carried a pregnancy to term, and when not desiring pregnancy controlled their menstrual symptoms with oral contraceptives or MPA. Longer duration of follow-up of the women in the Pittsburgh CHARM cohort will be necessary to more completely characterize the postmenopausal disease burden associated with a history of PCOS.

6.1 Determining the Menopause Phenotype of PCOS

A research priority set by the ASRM/ESHRE is to define the menopause phenotype of PCOS(12). The participants of CHARM have been well characterized during the middle to late reproductive years. Our data suggests the type 2 diabetes is a predominant feature among postmenopausal women with a history of PCOS. Today in 2012 the average age of the women seen at CHARM III visit 1 would between 57 and 59 years. This middle to late postmenopausal age range (100) represents an ideal population to address this knowledge gap. A study to

evaluate hormonal and metabolic features, associated sequelae including of cardio- and cerebrovascular events, as well as evaluations of menopause quality of life and pelvic floor disorders or presence of urogynecologic symptoms that are associated with aging, diabetes, and obesity would provide a profile of PCOS in the postmenopausal woman. A follow-up study in this well characterized cohort would shed new insights on the long-term health risks and experiences of older women with the condition that could inform practitioners on appropriate management strategies to guard against these risks.

6.2 Knowledge, Attitudes, and Practices in Approaching the PCOS Patient

Cyclic menstrual function and ovulation are indicators of a woman's overall health. While PCOS becomes evident at menarche and manifests throughout a woman's reproductive life, the underlying metabolic features associated with the condition may be silent and not be the first consideration when seeking or administering care. The American College of Obstetricians and Gynecologists released new practice guidelines in 2009 for the clinical management of the PCOS patient that encourage providers to follow Level A recommendations focused on behavioral management for metabolic risks (5). However, it is unclear whether there is uptake among U.S. providers and if there are adequate systems and referral networks to provide supportive interventions for this high risk population aimed at mitigating risks of metabolic syndrome including, type 2 diabetes. Given the findings presented in this investigation regarding phenotypic changes associated with age among women with PCOS, the condition and associated health risks may escape detection in perimenopausal women. Evaluation the knowledge, attitudes, and practices of gynecologist/obstetricians, reproductive endocrinology and infertility, family and internal medicine specialists would provide information on the current practices and awareness of providers about the management of PCOS and the availability and access to program that could modify risk for complications inside and outside the reproductive window. Information garnered from such an investigation could then be used

to develop a more comprehensive and integrated approach to administer care to women with PCOS that would be holistically focused and include planning for healthy reproductive transitions (adolescence to pregnancy to menopause) and incorporate strategies for diabetes and CVD prevention.

In all, these areas of investigation are rich in opportunity for advancement along a professional course. The later represents a linkage of public health, medicine, research, and health care policy and administration to advance women's health. I look forward to the next phase of my career and to contributing to improved health outcomes for women and families in as a public health professional.

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8.0 TABLES

Table 4a. Subject characteristics at CHARM III visit 1, presented as mean (SD) or percentage (n)

Variable	All		Age <45 years		Age 45-49		Ages 50-54		Age ≥55	
	Cases (n=152)	Controls (n=169)	Cases (n=63)	Controls (n=42)	Cases (n=44)	Controls (n=51)	Cases (n=28)	Controls (n=45)	Cases (n=17)	Controls (n=31)
Age (yrs)	47.2*** (5.6)	49.5 (5.9)	41.9 (2.1)	41.9 (2.2)	47.5 (1.5)	47.8 (1.4)	52.4 (1.4)	52.6 (1.5)	57.3✦✦✦ (1.8)	58.1✦✦✦ (2.6)
BMI (kg/m^2)	33.0 *** (9.2)	28.2 (6.1)	33.5** (9.8)	28.7 (6.7)	32.1* (7.1)	28.6 (6.4)	32.8* (9.8)	27.8 (6.2)	33.7 (11.5)	27.5 (4.2)
Waist:Hip Ratio	0.84 *** (0.08)	0.80 (0.08)	0.83* (0.09)	0.80 (0.07)	0.85* (0.08)	0.81 (0.08)	0.85** (0.09)	0.79 (0.09)	0.83 (0.09)	0.81 (0.08)
Natural Menopause	12.6%* (19)	23.1% (39)	-	-	4.5% (2)	5.9% (3)	22.2%* (6)	42.2% (19)	64.7%✦✦✦ (11)	54.8%✦✦✦ (17)
Surgical Menopause	7.3% (11)	10.1% (17)	4.8% (3)	4.8% (2)	13.6% (6)	9.8% (5)	3.7% (1)	15.6% (7)	5.9% (1)	9.7% (3)
BP Medications[1]	24.1% (27)	19.4% (21)	21.3%₡ (10)	6.3% (2)	29.4% (10)	26.5% (9)	20.0% (4)	13.6% (3)	27.3% (3)	35.0% (7)
Diabetes Medications[2]	10.3%* (11)	3.9% (3)	9.3% (4)	6.3% (2)	9.4%₡ (3)	-	14.3% (3)	4.8% (1)	9.1% (1)	-
Insulin[3]	2.0% (2)	1.0% (1)	-	3.1% (1)	3.0% (1)	-	5.0% (1)	-	-	-
Statin Use	7.2% (11)	4.7% (8)	1.6% (1)	2.4% (1)	9.1% (4)	3.9% (2)	10.7% (3)	2.2% (1)	17.6% (3)	12.9% (4)
Education (>12yrs)	78.8% (119)	73.2% (123)	84.1% (53)	78.6% (33)	76.7% (33)	82.0% (41)	75.0% (21)	73.3% (33)	70.6% (12)	51.6%✦ (16)
Race (% White)	84.1% (127)	79.9% (135)	79.0% (49)	81.0% (34)	90.9%₡ (40)	76.5% (39)	82.1% (23)	82.2% (37)	88.2% (15)	80.6% (25)

BMI-Body Mass Index; [1] BP-Blood Pressure Medications, n=112 cases and 108 controls; [2]Diabetes medications, n=107 cases and 101 controls;[3] Insulin, n=101 cases and 101 controls

Pearson's $\chi 2$, Fisher's exact or Mann-Whitney-Case Control comparisons within strata; ***(p<0.001); ** (p<0.01), *(p<0.05), ₡ (bordered on statistical significance p=0.050 to 0.099); $\chi 2$ or Kruskal Wallis-comparisons between strata within cases or controls; ✦✦✦ (p<0.001), ✦✦ (p<0.01), ✦(p<0.05), ∈(bordered on statistical significance)

Table 4b. Cardiovascular disease risk factors and coronary artery calcification at CHARM III visit 1, presented as median (IQR) or percentage (n)

	All		Age <45 years		Age 45-49		Ages 50-54		Age ≥55	
Variable	Cases (n=152)	Controls (n=169)	Cases (n=63)	Controls (n=42)	Cases (n=44)	Controls (n=51)	Cases (n=28)	Controls (n=45)	Cases (n=17)	Controls (n=31)
Smoker	16.4%	14.2%	12.7%	21.4%	20.5%	11.8%	17.9%	13.3%	17.6%	9.7%
SBP (mm Hg)	118.0℗ (109.2, 127.0)	113.0 (107.0, 125.0)	114.0 (108.0, 122.0)	111.0 (104.7, 118.5)	120.0*** (113.0, 126.7)	110.0 (106.0, 122.0)	125.0 (110.5, 128.7)	114.0 (108.5, 126.0)	119.0✦ (107.5, 130.0)	123.0✦✦✦ (114.0, 130.0)
DBP (mm Hg)	78.0 (70.0, 82.0)	76.0 (69.0, 81.0)	75.0 (68.0, 80.0)	72.5 (67.7, 80.0)	78.5* (72.0, 83.0)	74.0 (68.0, 81.0)	80.0 (70.2, 82.7)	78.0 (69.5, 82.0)	78.0∈ (69.0, 80.0)	79.0 (72.0, 81.0)
Chol (mg/dL)	201.0 (179.0, 228.0)	205.0 (184.0, 228.7)	200.5 (177.7, 218.0)	193.0 (171.7, 220.0)	201.5 (180.0, 227.5)	198.0 (184.0, 226.0)	218.0 (189.0, 248.5)	216.0 (198.0, 249.0)	194.0 (169.5, 239.0)	218.0✦ (182.0, 236.0)
HDL (mg/dL)	50.1*** (41.0, 63.6)	54.7 (46.9, 66.2)	51.3 (42.4, 65.0)	52.6 (45.9, 58.8)	44.0** (39.2, 58.6)	54.0 (47.5, 64.8)	51.0℗ (36.7, 72.1)	60.0 (50.1, 70.4)	54.4 (48.4, 62.6)	52.9∈ (46.8, 70.9)
LDL (mg/dL)	124.7 (103.7, 145.7)	121.5 (98.8, 144.0)	119,8 (96.6, 138.4)	123.0 (103.6, 148.2)	135.2 (11.5, 150.4)	131.8 (101.0, 146.6)	122.7 (93.5, 136.7)	124.0 (100.7, 147.5)	120.7 (99.5, 145.2)	103.5 (94.3, 157.1)
Trig (mg/dL)	125.5* (82.7, 199.5)	101.0 (77.4, 147.7)	97.5 (73.7, 197.5)	95.0 (77.8, 123.2)	126.0* (91.2, 199.5)	100.0 (63.0, 137.0)	147.0℗ (88.0, 244.0)	119.0 (74.5, 155.5)	128.0 (71.0, 193.0)	115.0 (88.5, 176.5)
Insulin (μU/mL)	16.5*** (10.3, 25.5)	11.1 (8.5, 15.6)	18.8* (10.2, 27.0)	11.9 (9.0, 19.8)	16.2** (10.0, 25.2)	11.0 (8.8, 15.3)	16.4*** (12.6, 25.0)	10.2 (7.8, 14.7)	13.9 (8.5, 21.5)	11.4 (9.7, 15.6)
Glucose (mg/dL)	92.7 (86.0, 102.3)	91.0 (86.6, 98.0)	90.0 (86.0, 97.0)	89.0 (85.2, 96.2)	92.7 (85.2, 103.2)	90.0 (85.0, 96.0)	95.8℗ (88.7, 107.6)	93.6 (86.6, 98.4)	99.0∈ (86.7, 111.2)	93.8 (88.9, 100.0)
Type 2 Diab	12.5%**	3.6%	7.9%	4.8%	13.6%	3.9%	17.9%℗	4.4%	17.6%*	0
HOMA IR	3.8 *** (2.3, 6.4)	2.5 (1.9, 3.6)	4.1℗ (2.2, 6.1)	2.7 (2.0, 4.0)	3.7** (2.1, 6.8)	2.4 (1.9, 3.4)	4.3*** (3.0, 6.0)	2.3 (1.7, 3.4)	3.0✦ (2.0, 5.9)	2.8 (2.2, 3.6)
CAC Agatson Score	3.09*** (0,19.22)	0 (0, 3.78)	1.03 (0, 13.56)	0 (0, 3.26)	2.06* (0, 16.39)	0 (0, 4.46)	4.8*** (0.26, 56.56)	0 (0, 3.6)	19.91**,✦✦ (1.03, 66.08)	1.03 (0, 3.78)
Any CAC	63.1%***	41.7%	55.0%℗	38.1%	59.1%℗	42.0%	75.0%**	37.8%	82.4%*,∈	51.6%
CAC ≥ 10	35.6%***	14.9%	30.7%	20.9%	31.8%**	10.0%	42.9%**	13.3%	58.8%**,∈	16.2%

IQR-Interquartile Range; SBP-Systolic Blood Pressure; DBP-Diastolic Blood Pressure; HDL-High Density Lipoprotein; LDL-Low Density Lipoprotein; HOMA-Homeostatic Model Assessment; HOMA-IR-Insulin Resistance; CAC-Coronary Artery Calcification

Pearson's $\chi 2$, Fisher's exact or Mann-Whitney-Case Control comparisons within strata; ***(p<0.001); ** (p<0.01), *(p<0.05), ℗ (bordered on statistical significance p=0.050 to 0.099); $\chi 2$ or Kruskal Wallis-comparisons between strata within cases or controls; ✦✦✦ (p<0.001), ✦✦ (p<0.01), ✦(p<0.05), ∈(bordered on statistical significance)

Table 4c. Reproductive hormones obtained at CHARM III visit 1, presented as median (IQR) or percentage (n)

	All		Age <45 years		Age 45-49		Ages 50-54		Age ≥55	
Variable	Cases (n=152)	Controls (n=169)	Cases (n=61)	Controls (n=42)	Cases (n=44)	Controls (n=51)	Cases (n=28)	Controls (n=44)	Cases (n=17)	Controls (n=29)
Current OCs	7.9% (12)	5.9% (10)	11.1% (7)	11.9% (5)	6.8% (3)	9.8% (5)	7.4% (2)	-	-	-
Current HRT	11.8% (18)	18.9% (32)	1.6% (1)	4.9% (2)	18.2% (8)	9.8% (5)	17.9% (5)	28.9% (13)	23.5% (4)	38.7% (12)
LH IU/L	9.5*** (4.8, 18.5)	17.2 (5.0, 36.4)	6.5 (3.8, 10.5)	4.8 (2.6, 7.7)	8.4 (3.8, 20.8)	8.0 (4.3, 23.3)	13.9*** (8.1, 22.9)	32.3 (16.3, 50.4)	25.3***, ✦✦✦ (12.3, 30.5)	38.8 ✦✦✦ (28.8, 52.3)
FSH IU/L	8.0*** (5.0, 22.9)	25.9 (6.4, 62.2)	5.3 (4.2, 6.8)	5.8 (3.9, 10.8)	9.3 (5.2, 18.2)	12.1 (5.6, 32.3)	18.5*** (8.3, 36.4)	56.7 (26.6,90.4)	37.3***, ✦✦✦ (24.6, 44.1)	59.3✦✦✦ (43.6, 93.8)
LH/FSH	0.81*** (0.61, 1.29)	0.62 (0.47, 0.85)	1.25*** (0.71, 1.75)	0.76 (0.53, 1.12)	0.79* (0.60, 1.08)	0.63 (0.45, 0.83)	0.73* (0.55, 0.98)	0.55 (0.45, 0.75)	0.64 ✦✦✦ (0.45, 0.73)	0.61∈ (0.41, 0.75)
E2 pg/mL	59.5 (37.7, 102.3)	57.1 (31.7, 113.6)	71.9 (48.9, 128.8)	81.1 (44.4, 115.0)	57.3 (47.5, 96.8)	62.6 (36.4, 141.2)	51.0 (31.2, 83.5)	49.7 (28.0, 102.9)	33.7 ✦ (26.0, 61.0)	31.0✦ (20.0, 93.3)
TT ng/dL	23.0** (19.9, 40.3)	20.2 (19.9, 28.8)	25.9* (19.9, 43.2)	20.1 (19.9, 26.6)	20.2 (19.9, 39.6)	23.0 (19.9, 28.8)	21.6 (19.9, 43.9)	23.0 (19.9, 27.4)	31.7¶ (19.9, 40.3)	19.9 (19.9, 28.8)
SHBG nmol/L	85.4*** (56.2, 169.9)	152.7 (92.3, 230.0)	85.6* (54.8, 164.2)	144.7 (89.9, 249.7)	84.3*** (62.4, 169.9)	170.4 (107.9, 236.4)	79.1¶ (40.1, 204.8)	147.9 (75.5, 214.2)	86.9 (56.9, 151.6)	119.4 (80.0, 231.8)
FAI	1.11*** (0.51, 2.08)	0.57 (0.38, 0.91)	1.10** (0.51, 2.29)	0.55 (0.40, 0.83)	0.93*** (0.51, 1.79)	0.55 (0.33, 0.84)	1.07* (0.44, 2.78)	0.59 (0.41, 0.97)	1.30** (0.66, 1.81)	0.67 (0.33, 1.23)
FEI	0.28*** (0.12, 0.46)	0.15 (0.08, 0.26)	0.34* (0.17, 0.52)	0.19 (0.11, 0.34)	0.24* (0.13, 0.40)	0.16 (0.07, 0.26)	0.27¶ (0.09, 0.57)	0.12 (0.08, 0.25)	0.14∈ (0.07, 0.34)	0.11✦ (0.05, 0.16)
FAI/FEI	5.0* (2.8, 8.0)	3.9 (2.1, 7.6)	4.4 (2.6, 5.9)	2.8 (2.1, 6.4)	4.5 (2.69, 7.74)	3.48 (1.94, 5.69)	6.5 (3.2, 9.7)	4.7 (2.2, 8.4)	7.5✦✦ (5.4, 11.2)	7.3∈ (2.6, 12.6)

IQR-Interquartile Range; MPA-Medroxyprogesterone Acetate; OCs-Oral Contraceptives; HRT-Hormone Replacement Therapy; LH-Luteinizing Hormone; FSH-Follicle Stimulating Hormone; E2-estradiol; TT-Total Testosterone; SHBG-Sex Hormone Binding Globulin; FAI-Free Androgen Index; FEI-Free Estradiol Index Pearson's $\chi 2$, Fisher's exact or Mann-Whitney-Case Control comparisons within strata; ***(p<0.001); ** (p<0.01), *(p<0.05), ¶ (bordered on statistical significance p=0.050 to 0.099); Kruskal Wallis-comparisons between strata within cases or controls; ✦✦✦ (p<0.001), ✦✦ (p<0.01), ✦(p<0.05), ∈(bordered on statistical significance)

Table 5. Reproductive profile of participants at CHARM III visit 1, presented as mean (SD) or n (%)

Variable	Cases (n=152)	Controls (n=169)	p
Age (years)	47.2 (5.6)	49.5(5.9)	**<0.001**
Age at Menarche (years)	12.8 (1.9)	12.8 (1.7)	0.701
Ever Pregnant	113 (74.8%)	136 (80.5%)	0.266
Number of Pregnancies	2.6 (1.6)	2.7 (1.4)	0.156
Number of Live Births	1.6 (1.2)	2.2(1.2)	**<0.001**
Fertility Medications	98 (64.9%)	14 (8.3%)	**<0.001**
Ever Used OCs	120 (81.1%)	132 (78.6%)	0.580
Current OC Use	10 (6.7%)	10 (5.9%)	0.783
Ever Used HRT	32 (21.6%)	54 (32.1%)	**0.036**
Current HRT Use	18 (11.9%)	31 (18.5%)	0.101
Natural Menopause	19 (12.6%)	39 (23.1%)	**<0.017**
Surgical Menopause	11 (7.3%)	17 (10.1%)	0.381
BSO and/or Hysterectomy	22 (14.5%)	34 (20.4%)	0.680

OCs-Oral Contraceptive; HRT-Hormone Replacement Therapy; BSO-Bilateral salpingo-oophorectomy
P-value by $\chi 2$ or Mann-Whitney U-test

Table 6a. CHARM III, visit 1 characteristics of cases and controls by menopausal group presented as mean (SD), median (IQR), or percentage (n)

Group	Not Menopausal		Menopausal		Surgical Menopause		Unknown	
Variable	Cases 55%(106)	Controls 45%(86)	Cases 31%(24)	Controls 69%(54)	Cases 41%(14)	Controls 59%(20)	Cases 47%(8)	Controls 53%(9)
Age (yrs)	45.0 (4.4)	45.7 (4.2)	55.2 (3.4)	54.8 (4.0)	47.7∉ (4.1)	51.2 (5.7)	50.4 ✦✦✦ (3.2)	50.4✦✦✦ (2.7)
BMI (kg/m^2)	33.2*** (9.4)	28.3 (6.3)	33.1** (9.2)	27.4 (5.7)	31.5 (8.3)	29.9 (6.6)	32.4✦ (9.9)	28.0 (4.2)
Waist:Hip Ratio	0.84*** (0.08)	0.80 (0.07)	0.88*** (0.08)	0.80 (0.08)	0.83 (0.09)	0.82 (0.08)	0.79 (0.09)	0.81 (0.09)
Currently Smoking	17.0% (18)	12.8% (11)	20.8% (5)	13.0% (7)	7.1% (1)	25.0% (5)	12.5% (1)	11.1% (1)
SBP (mm Hg)	117.0** (109.0, 125.0)	111.0 (106.0, 120.0)	121.5 (107.7, 131.0)	119.0 (109.0, 128.0)	122.5 (109.0, 130.2)	117.5 (111.2, 128.5)	121.5 (113.0, 127.7)	109.0✦ (108.0, 143.0)
DBP (mm Hg)	78.0∉ (70.0, 82.0)	73.5 (68.7, 80.0)	78.0 (68.5, 80.0)	76.5 (69.0, 82.1)	79.5 (70.7, 85.2)	79.0 (69.7, 82.7)	77.5 (72.0, 82.2)	77.0 (66.5, 81.5)
Cholesterol (mg/dL)	202.0 (179.0, 227.0)	199.5 (182.0, 224.0)	195.5 (173.7, 241.5)	216.0 (191.0, 243.5)	217.0 (186.5, 252.5)	213.5 (175.5, 225.0)	192.5 (170.7, 197.0)	200.0 (168.0, 247.0)
HDL (mg/dL)	47.0** (39.9, 62.8)	53.9 (46.8, 65.0)	53.6 (45.4, 57.8)	55.9 (47.5, 69.7)	50.7 (45.0, 69.6)	54.4 (46.9, 62.5)	52.0✦ (42.5, 72.4)	63.7 (47.5, 72.8)
LDL (mg/dL)	123.6 (102.7, 144.0)	122.8 (106.3, 146.5)	103.9∉ (92.5, 144.4)	135.2 (109.5, 148.1)	126.7 (95.5, 160.1)	117.0 (97.7, 136.9)	106.1∉ (94.1, 111.7)	121.7 (86.7, 156.8)
Triglyceride (mg/dL)	115.0** (77.5, 198.0)	95.0 (71.7, 129.2)	139.0 (87.5, 242.5)	127.0 (81.0, 163.5)	121.0 (92.5, 197.0)	125.5 (102.2, 191.5)	103.5 (79.0, 174.0)	113.0✦✦ (75.0, 137.5)
Insulin (μU/mL)	17.8*** (10.0, 25.6)	11.2 (8.7, 16.0)	18.0*** (12.3, 27.8)	10.4 (8.5, 14.3)	14.0 (9.9, 27.0)	11.7 (8.1, 19.2)	13.3✦ (10.1, 16.6)	11.4 (8.1, 19.3)
Glucose (mg/dL)	91.0 (86.0, 100.6)	90.0 (85.0, 97.2)	104.5** (89.5, 122.9)	93.6 (87.6, 99.0)	92.7 (85.0, 96.9)	92.8 (85.5, 98.7)	93.3✦✦✦ (88.7, 97.1)	88.0 (87.1, 94.0)
Type 2 Diabetes	8.5% (9)	4.7% (4)	25.0%** (6)	1.9% (1)	28.6%∉ (4)	5.0% (1)	-	-
HOMA-IR	4.2*** (2.0, 6.3)	2.6 (1.9, 4.0)	4.8*** (2.9, 9.9)	2.4 (1.9, 3.4)	2.9 (2.2, 6.7)	2.5 (1.8, 4.5)	3.1✦✦ (2.2, 3.9)	2.5 (1.7, 4.9)
CAC Agatson Score	1.7*** (0, 14.4)	0 (0, 3.6)	20.1*** (1.0, 61.4)	0 (0, 7.7)	4.8* (0, 35.1)	0.5 (0, 3.4)	0.7✦✦ (0, 9.2)	1.4 (0, 4.8)
Any CAC	59.2%** (61)	37.6% (32)	79.2%** (19)	42.6% (23)	74.1% (10)	50.0% (10)	50.0% (4)	55.6% (5)
CAC ≥ 10	30.1%** (31)	12.9% (11)	58.3%** (14)	22.2% (12)	42.9%* (6)	5.0% (1)	25.0% (2)	11.1% (1)

IQR-Inter Quartile Range; SBP-Systolic Blood Pressure; DBP-Diastolic Blood Pressure; HDL-High Density Lipoprotein; LDL-Low Density Lipoprotein; HOMA-Homeostatic Model Assessment; HOMA-IR-Insulin Resistance; CAC-Coronary Artery Calcification ; Pearson's $\chi 2$, Fisher's exact or Mann-Whitney-Case Control comparisons within strata; ***(p<0.001); ** (p<0.01), *(p<0.05), ∉ (bordered on statistical significance p=0.050 to 0.099); Kruskal Wallis-comparisons between strata within cases or controls; ✦✦✦ (p<0.001), ✦✦ (p<0.01), ✦(p<0.05), ∈(bordered on statistical significance)

Table 6b. CHARM III, visit 1 reproductive characteristics of cases and controls by menopausal group presented as median (IQR) or percentage (n)

Group	Not Menopausal		Menopausal		Surgical Menopause		Unknown	
Variable	Cases 55%(106)	Controls 45%(86)	Cases 31%(24)	Controls 69%(54)	Cases 41%(14)	Controls 59%(20)	Cases 47%(8)	Controls 53%(9)
Current OC	9.5% (10)	8.1% (7)	-	-	-	-	33.3% (3)	25.0% (2)
Duration OCs (months)	24.0 (6.0, 72.0)	48.0 (5.2, 108.0)	6.0 (0, 33.0)	12.0 (0, 111.0)	12.0 (0.7, 25.5)	8.0 (0, 51.0)	36.0 (0.7, 156.0)	72.0 (54.0, 172.5)
Current HRT	-	-	20.8% (5)	27.8% (15)	57.1% (8)	57.9% (11)	62.5% (5)	66.7% (6)
Duration HRT (months)	-	-	0 (0, 21.0)	4.0 (0, 60.0)	36.0 (0, 111.0)	6.0 (0, 60.0)	0 (0, 5.2)	8.0 (0, 66.0)
Duration Menopause (mo)	-	-	39.5 (31.0, 82.1) n=15	59.3 (18.8, 96.9) n=36	62.7 (43.6, 275.5) n=8	96.5 (51.4, 133.3) n=18	-	-
LH IU/L	7.0 (3.8, 12.2)	5.6 (3.5, 14.5)	25.4*** (14.0, 29.5)	38.7 (28.8, 55.3)	17.7 (10.9, 27.4)	32.2 (17.5, 48.7)	5.7✦✦✦ (3.8, 11.4)	9.4✦✦✦ (3.9, 12.0)
FSH IU/L	5.9¶ (4.5, 9.6)	7.0 (4.4, 24.4)	38.0*** (24.9, 45.1)	74.7 (48.2, 96.8)	25.1* (13.4, 40.8)	48.1 (35.1, 64.0)	7.7✦✦✦ (5.6, 10.5)	13.4✦✦✦ (4.5, 25.6)
LH:FSH	0.95*** (0.67, 1.59)	0.74 (0.51, 1.03)	0.63¶ (0.48, 0.78)	0.55 (0.44, 0.70)	0.74 (0.53, 0.98)	0.61 (0.50, 0.74)	0.72✦✦✦ (0.47, 0.99)	0.58✦✦✦ (0.32, 0.91)
E2 pg/mL	71.5 (49.6, 119.2)	76.8 (45.1, 142.3)	36.6 (25.5, 61.9)	32.9 (21.1, 60.7)	49.1 (26.8, 57.2)	53.7 (29.4, 119.9)	46.5✦✦✦ (22.6, 77.2)	85.3✦✦✦ (42.7, 156.7)
TT ng/dL	24.5¶ (19.9, 42.5)	23.0 (19.9, 28.8)	31.7 (19.9, 42.5)	20.2 (19.9, 28.8)	21.6 (19.9, 41.8)	21.6 (19.9, 28.1)	19.9 (19.9, 31.7)	19.9 (19.9, 20.0)
SHBG nmol/L	79.0*** (56.3, 160.0)	158.2 (100.1, 224.8)	85.9* (55.6, 163.5)	132.7 (79.0, 210.8)	100.0 (55.1, 206.7)	155.7 (61.6, 299.0)	132.7¶, ✦✦✦ (63.8, 217.8)	214.8✦✦✦ (117.2, 300.2)
FAI	1.06*** (0.51, 2.19)	0.61 (0.42, 0.84)	1.32** (0.69, 1.97)	0.63 (0.41, 0.98)	1.22 (0.34, 2.56)	0.50 (0.24, 1.46)	0.72¶, ✦✦✦ (0.33, 1.22)	0.32✦✦✦ (0.23, 0.59)
FEI	0.32** (0.14, 0.52)	0.19 (0.11, 0.33)	0.18** (0.07, 0.31)	0.10 (0.05, 0.16)	0.18 (0.09, 0.36)	0.17 (0.09, 0.26)	0.28✦✦✦ (0.04, 0.40)	0.17✦✦✦ (0.06, 0.47)
FAI/FEI	4.2* (2.4, 6.4)	2.9 (2.0, 5.5)	8.6 (5.5, 11.0)	7.4 (3.8, 11.7)	6.4 (4.4, 13.6)	4.7 (1.9, 6.6)	5.1¶,✦✦✦ (2.5, 9.7)	2.2✦✦✦ (1.2, 4.5)

IQR-Inter Quartile Range; HRT/OCs-current Hormone Replacement Therapy or Oral Contraceptives; LH-Luteinizing Hormone; FSH- Follicle Stimulating Hormone; E2-Estradiol; TT-Total Testosterone; SHBG-Sex Hormone Binding Globulin; FAI-Free Androgen Index; FEI-Free Estradiol Index

Pearson's $\chi 2$, Fisher's exact or Mann-Whitney-Case Control comparisons within strata; ***(p<0.001); ** (p<0.01), *(p<0.05), ¶ (bordered on statistical significance p=0.050 to 0.099); Kruskal Wallis-comparisons between strata within cases or controls; ✦✦✦ (p<0.001), ✦✦ (p<0.01), ✦(p<0.05), ∈(bordered on statistical significance)

Table 7. Estimated parameters, adjusted R^2 from multiple linear regression equations for predictors of cardiovascular disease risk factors that differed between cases and controls

	Models	Independent variable		
		SBP	HDL	Trig*
1	PCOS β (95% CI) (p value) Adjusted R^2	1.642 (-1.229, 4.513) p=0.261 0.001	-5.061 (-8.372, -1.751) **p=0.003** 0.026	0.178 (0.048, 0.309) **p=0.007** 0.020
2	PCOS	0.538 (-2.270, 3.346) p=0.706	-1.771 (-5.109, 1.567) p=0.297	0.128 (-0.007, 0.260) p=0.058
	Age	0.563 (0.338, 0.788) **p<0.001**	0.248 (-0.021, 0.517) p=0.071	0.017 (0.006, 0.027) **p=0.002**
	BMI	0.519 (0.349, 0.689) **p<0.001**	-0.573 (-0.775, -0.371) **p<0.001**	0.019 (0.011, 0.027) **p<0.001**
	Adjusted R^2	0.157	0.123	0.104
3	PCOS	0.490 (-2.334, 3.313) p=0.733	-1.729 (-5.093, 1.634) p=0.312	0.139 (0.008, 0.271) **p=0.038**
	Age	0.629 (0.346, 0.687) **p<0.001**	0.229 (-0.136, 0.594) p=0.218	0.010 (-0.004, 0.024) p=0.166
	BMI	0.517 (0.346, 0.687) **p<0.001**	-0.574 (-0.777, -0.371) **p<0.001**	0.019 (0.011, 0.027) **p<0.001**
	Not Menopausal	0	0	0
	Natural Menopause	-1.561 (-5.826, 2.705) p=0.472	0.279 (-4.808, 5.366) p=0.914	0.113 (-0.086, 0.313) p=0.265
	Surgical Menopause	1.630 (-2.864, 6.123) p=0.476	1.280 (-4.070, 6.629) p=0.638	0.275 (0.065, 0.485) **p=0.010**
	Adjusted R^2	0.156	0.118	0.118

*Trig-Triglyceride levels were log transformed prior to entry into model.
BMI-Body Mass Index; SBP-Systolic Blood Pressure; HDL-High Density Lipoprotein cholesterol

Table 7 continued

Model		Independent Variable		
		SBP	HDL	Trig*
4	PCOS	0.760 (-2.685, 4.205) p=0.665	-1.852 (-5.957, 2.253) p=0.375	0.155 (-0.006, 0.316) p=0.058
	Age	0.628 (0.322, 0.934) **p<0.001**	0.229 (-0.136, 0.595) p=0.218	0.010 (-0.004, 0.024) p=0.168
	BMI	0.516 (0.346, 0.687) **p<0.001**	-0.573 (-0.777, -0.370) **p<0.001**	0.019 (0.011, 0.027) **p<0.001**
	Not Menopausal	0	0	0
	Natural Menopause	-1.250 (-6.087, 3.588) p=0.612	0.136 (-5.642, 5.914) p=0.963	0.132 (-0.095, 0.358) p=0.253
	Surgical Menopause	1.991 (-3.226, 7.209) p=0.453	1.115 (-5.099, 7.329) p=0.724	0.296 (0.053, 0.540) **p=0.017**
	PCOS* Menopause	-0.781 (-6.479, 4.916) p=0.787	0.356 (-6.440, 7.153) p=0.918	-0.046 (-0.313, 0.220) p=0.732
	Adjusted R^2	0.154	0.115	0.116
5	PCOS	0.679 (-2.764, 4.121) p=0.698	-2.086 (-6.164, 1.992) p=0.315	0.147 (-0.012, 0.306) p=0.069
	Age	0.650 (0.343, 0.956) **p<0.001**	0.236 (-0.128, 0.600) p=0.204	0.012 (-0.002, 0.026) p=0.097
	BMI	0.531 (0.360, 0.703) **p<0.001**	-0.541 (-0.744, -0.337) **p<0.001**	0.020 (0.012, 0.028) **p<0.001**
	Not Menopausal	0	0	0
	Natural Menopause	-1.857 (-6.756, 3.042) p=0.456	-0.935 (-6.747, 4.876) p=0.752	0.074 (-0.155, 0.302) p=0.519
	Surgical Menopause	0.380 (-5.270, 6.029) p=0.895	-1.847 (-8.536, 4.843) p=0.587	0.140 (-0.153, 0.439) p=0.290
	PCOS* Menopause	-0.367 (-6.074, 5.341) p=0.899	0.660 (-6.106, 7.426) p=0.848	-0.011 (-0.311, 0.296) p=0.937
	Taking Hormones	1.662 (-2.076, 5.401) p=0.382	5.819 (1.363, 10.275) **p=0.011**	0.182 (0.008, 0.356) **p=0.040**
	Adjusted R^2	0.156	0.131	0.129

*Trig-Triglyceride levels were log transformed prior to entry into model.
BMI-Body Mass Index; SBP-Systolic Blood Pressure; HDL-High Density Lipoprotein cholesterol

Table 8. Logistic regression models of CAC (<10 vs. ≥10) between PCOS cases and controls (n=300)

Models	β	SE	OR	95% CI for OR	p Value
1. PCOS	1.159	0.282	3.187	1.832, 5.545	<0.001
2. PCOS	0.778	0.338	2.176	1.121, 4.223	0.022
Age	0.060	0.028	1.061	1.055, 1.120	0.031
Body Mass Index	0.173	0.025	1.189	1.132, 1.248	<0.001
3. PCOS	1.384	0.304	3.991	2.200, 7.240	<0.001
Not Menopausal (n=188)			1		
Natural Menopause (n=78)	0.961	0.329	2.614	1.371, 4.985	0.004
Surgical Menopause (n=34)	0.079	0.478	1.082	0.424, 2.763	0.869
4. PCOS	0.869	0.353	2.385	1.195, 4.762	0.014
Age	-0.009	0.038	0.991	0.920, 1.068	0.814
BMI	0.183	0.026	1.201	1.141, 1.264	<0.001
Not Menopausal			1		
Natural Menopause	1.519	0.536	4.570	1.599, 13.060	0.005
Surgical Menopause	0.089	0.566	1.093	0.361, 3.315	0.875
5. PCOS	0.433	0.456	1.541	0.631, 3.766	0.342
Age	-0.005	0.038	0.995	0.923, 1.073	0.899
BMI	0.184	0.026	1.202	1.142, 1.266	<0.001
Not Menopausal			1		
Natural Menopause	0.985	0.645	2.679	0.757, 9.483	0.126
Surgical Menopause	-0.541	0.726	0.582	0.140, 2.418	0.457
PCOS*Menopause	1.001	0.702	2.722	0.688, 10.771	0.154

BMI-Body Mass Index; CAC-Coronary Artery Calcification

Table 9. Descriptive characteristics of participants at CHARM III visit 1 matched for age and race, presented as mean (SD) or percentage (n)

Variable	Cases (n=83)	Controls (n=83)
Age (years)	47.6 (5.8)	47.6 (5.6)
BMI (kg/m^2)	33.5*** (9.5)	27.5 (5.6)
Waist:Hip Ratio	0.85*** (0.08)	0.79 (0.07)
Age at Menarche (years)	13.1 (2.2)	12.7 (1.6)
Not Menopausal	73.5% (61)	81.9% (68)
Natural Menopause	18.1% (15)	27.7% (23)

Pearson $\chi 2$ or Mann-Whitney ***(p<0.001); ** (p<0.01), *(p<0.05), ¢ (bordered on statistical significance p=0.050 to 0.099)

Table 10. Subject characteristics at CHARM III visit 1, presented as mean (SD) or percentage (n)

Variable	Not Menopausal		Menopausal	
	Cases 53%(68)	Controls 47%(60)	Cases 44%(18)	Controls 56%(23)
Age (yrs)	45.9 (4.5)	45.2 (4.2)	55.6 (3.5)	54.1 (3.6)
BMI (kg/m^2)	33.1*** (9.4)	27.3 (4.8)	35.4* (10.3)	28.1 (7.2)
Waist:Hip Ratio	0.84*** (0.08)	0.79 (0.07)	0.89** (0.08)	0.80 (0.09)
Age at Menarche (yrs)	13.3 (2.2)	12.5 (1.3)	12.3 (2.1)	13.0 (2.3)
Duration of Menopause (months)	-	-	61.5 (58.5)	60.5 (50.4)
LH	10.9¶ (8.7)	11.3 (14.9)	23.1*** (11.0)	50.2 (22.0)
FSH	11.5 (13.0)	17.3 (31.0)	35.5*** (15.5)	94.5 (41.0)
LH:FSH Ratio	1.21** (0.77)	0.95 (0.91)	0.67* (0.17)	0.54 (0.12)
Estradiol	97.4 (62.9)	113.7 (109.4)	40.4 (20.1)	31.0 (14.4)
Total Testosterone	36.1 (32.5)	26.1 (10.0)	37.7 (20.2)	29.4 (14.5)
SHBG	129.6*** (125.5)	162.9 (83.9)	94.6* (74.6)	139.5 (77.9)
FAI	1.66*** (1.60)	0.70 (0.44)	2.62** (3.78)	0.92 (0.61)
FEI	0.47** (0.48)	0.29 (0.25)	0.28** (0.32)	0.10 (0.06)
FAI/FEI	4.44¶ (3.21)	3.39 (2.40)	9.38 (3.34)	9.91 (4.80)

Mann-Whitney-Case Control comparisons within strata; ***(p<0.001); ** (p<0.01), *(p<0.05), ¶ (bordered on statistical significance p=0.050 to 0.099)

Table 11. Cardiovascular disease risk factors and coronary artery calcification at CHARM III visit 1, presented as mean (SD) or percentage (n)

	Not Menopausal		Menopausal	
Variable	Cases 53%(68)	Controls 47%(60)	Cases 44%(18)	Controls 56%(23)
Smoker	23.5% (16)	15.0% (9)	20.0% (3)	13.0% (3)
SBP (mm Hg)	117.9*** (11.0)	112.5 (14.3)	120.2 (13.8)	118.5 (12.9)
DBP (mm Hg)	76.0* (8.4)	72.7 (8.4)	75.1 (10.2)	74.7 (8.4)
Chol (mg/dL)	208.9 (42.8)	203.2 (35.0)	212.3 (44.1)	211.9 (34.1)
HDL (mg/dL)	50.3** (16.9)	55.3 (12.0)	53.8 (13.6)	55.1 (14.8)
LDL (mg/dL)	130.28 (35.3)	126.7 (30.0)	120.2 (34.8)	129.7 (32.3)
Trig (mg/dL)	148.0¶ (108.1)	105.8 (48.5)	205.2 (193.6)	129.6 (76.6)
Insulin (μU/mL)	18.7*** (10.8)	11.7 (4.6)	23.0** (10.9)	15.0 (10.9)
Glucose (mg/dL)	94.9 (14.0)	96.3 (37.5)	119.3* (40.9)	93.0 (10.5)
Type 2 Diab	10.3%¶ (7)	1.7% (1)	33.3%** (5)	0
HOMA IR	4.6*** (3.3)	2.9 (2.8)	7.2*** (4.6)	3.6 (3.0)
CAC Agatson Score	13.4*** (31.7)	5.4 (26.6)	119.7* (249.7)	18.1 (46.1)
Any CAC	60.6%*** (40)	30.0% (18)	80.0%* (12)	39.1% (9)
CAC ≥ 10	25.7%* (17)	10.0% (6)	60.0%* (9)	26.1% (6)

Pearson $\chi 2$, Fisher's exact, or Mann-Whitney-Case Control comparisons within strata; ***($p<0.001$); ** ($p<0.01$), *($p<0.05$), ¶ (bordered on statistical significance p=0.050 to 0.099

Table 12. CHARM III, Visit 1 number of cycles/years reported by cases and control when not using hormones or pregnant, presented a median (IQR)

	Teens	20s	30s	40s	Total
Cases	8.0 (4.0, 12.0) n=134	5.0 (2.4, 9.2) n=134	8.8 (4.3,11.8) n=133	12.0 (9.1, 12.0) n=119	7.9 (5.3, 9.7) n=132
Controls	12.0 (10.3, 12.0) n=147	8.6 (2.8, 11.0) n=148	11.0 (9.4, 12.0) n=148	12.0 (11.0, 12.0) n=136	10.0 (7.9, 11.1) n=145
p value*	<0.001	0.002	<0.001	0.006	<0.001

*Mann-Whitney

Table 13. Characteristics of women with PCOS at CHARM III visit 1, stratified by adjusted cumulative cycle number per reproductive years contributed, shown as mean (SD) or percent (n)

Variable	< 9 cycles n=53	9-11 cycles/year n=37	>11 cycles/year n=48	p*
Adjusted Cycle #/ yr	6.5 (1.8)	10.0 (0.52)	12.0 (0.86)	**<0.001**
Natural Cycle #/yr	5.2 (1.9)	7.9 (2.2)	9.5 (2.5)	**<0.001**
Age at visit (yrs)	46.6 (6.1)	47.6 (5.9)	46.5 (4.4)	0.685
BMI (kg/m^2)	33.6 (8.4)	33.3 (9.3)	32.4 (10.1)	0.614
Waist:Hip ratio	0.85 (0.09)	0.84 (0.07)	0.83 (0.09)	0.674
Education (>12 yrs.)	75.0% (39)	91.9% (34)	79.2% (38)	0.124
Race (% White)	81.1% (43)	77.8% (28)	89.6% (43)	0.313
Menarche (yrs)	13.2 (2.2)	12.4 (1.5)	12.7 (1.9)	0.316
Period last 12 mos.	77.4% (41)	73.0% (27)	75.0% (36)	0.891
Natural Menopause	17.0% (9)	8.3% (3)	4.2% (2)	0.095
Surgical Menopause	5.7% (3)	2.8% (1)	10.4% (5)	0.343
Ever Pregnant	75.5% (40)	81.1% (30)	72.9% (35)	0.676
Fertility Treatments	71.7% (38)	56.8% (21)	62.5% (30)	0.363
# Pregnancies	2.4 (1.2)	2.6 (2.0)	2.9 (1.7)	0.535
# Live Births	1.7 (0.9)	1.7 (1.3)	1.5 (1.4)	0.479
Current OCs	7.5% (4)	8.1% (3)	10.4% (5)	0.868
Current HRT	5.7% (3)	13.5% (5)	14.6% (7)	0.296
LH (IU/L)	12.0 (8.8)	12.9 (9.7)	12.9 (14.4)	0.424
FSH (IU/L)	13.3 (12.8)	17.1 (15.0)	15.6 (20.4)	0.244
LH/FSH	1.1 (0.67)	1.0. (0.6)	1.0 (0.7)	0.395
E2 (pg/mL)	81.3 (76.7)	77.9 (60.0)	86.1 (65.8)	0.584
TT (ng/dL)	55.3 (97.9)	30.6 (14.9)	27.7 (13.5)	**0.003**
SHBG (nmol/L)	120.3 (103.7)	131.8 (124.3)	151.5 (144.5)	0.460
FAI	2.60 (3.48)	1.45 (1.31)	1.15 (1.11)	**0.015**
FEI	0.38 (0.32)	0.41 (0.51)	0.36 (0.40)	0.697
FAI:FEI ratio	8.89 (16.5)	5.79 (3.94)	5.07 (4.58)	**0.014**
Current Smoker	13.2% (7)	13.5% (5)	22.9% (11)	0.355
Type 2 Diabetes	13.2% (7)	8.1% (3)	10.4% (5)	0.741
Insulin ($\mu U/mL$)	20.9 (12.0)	18.3 (11.1)	18.2 (12.7)	0.382
Glucose (mg/dL)	101.8 (28.9)	95.2 (21.1)	94.8 (13.2)	0.394
HOMA-IR	5.7 (4.9)	4.5 (3.3)	4.5 (3.7)	0.362
SBP (mm Hg)	117.3 (10.9)	118.6 (11.5)	118.1 (10.5)	0.852
DBP (mm Hg)	76.3 (8.5)	77.1 (8.1)	75.1 (7.4)	0.589
Cholesterol (mg/dL)	215.7 (51.5)	205.1 (37.5)	201.2 (35.4)	0.548
HDL (mg/dL)	51.3 (15.3)	52.6 (17.9)	53.7 (14.3)	0.621
LDL (mg/dL)	131.8 (49.1)	119.2 (34.3)	122.0 (29.2)	0.610
Triglyceride (mg/dL)	170.7 (133.4)	161.5 (124.7)	127.0 (75.8)	0.157
CAC Agatson Score	40.5 (142.8)	17.9 (27.3)	15.3 (29.1)	0.319
Any CAC	67.3% (35)	67.6% (25)	52.2% (24)	0.223
CAC ≥ 10	34.6% (18)	45.9% (17)	28.3% (13)	0.243

HRT- Hormone Replacement Therapy; OCs- Oral Contraceptives; BMI-Body Mass Index; LH-Luteinizing Hormone; FSH-Follicle Stimulating Hormone; E2-Estradiol; TT-Total Testosterone; SHBG-Sex Hormone Binding Globulin;FAI-Free Androgen Index; FEI-Free Estradiol Index; SBP-Systolic Blood Pressure; DBP-Diastolic Blood Pressure; HDL-High Density Lipoprotein; LDL-Low Density Lipoprotein; HOMA-IR-Homeostatic Model Assessment Insulin Resistance; CAC-Coronary Artery Calcification

*$\chi 2$ or Kruskal-Wallis for p values

Table 14. Logistic regression models of Coronary Artery Calcification (<10 vs. ≥10) in PCOS cases (n=142) using lifetime irregularity as a predictor

Models		β	SE	OR	95% CI for OR	p Value
1.	Total Adjusted Cycles (TAC)	0.001	0.002	1.001	0.998, 1.004	0.519
2.	TAC	0.000	0.002	1.000	0.996, 1.003	0.880
	Age	0.084	0.034	1.088	1.018, 1.162	0.013
3.	TAC	0.001	0.002	1.001	0.997, 1.005	0.580
	Age	0.102	0.038	1.107	1.029, 1.192	0.007
	*HOMA-IR	1.263	0.303	3.535	1.950, 6.406	<0.001

*HOMA-IR-Homeostatic Model Assessment Insulin Resistance; log transformed values used in model

Table 15. Selected outcomes in PCOS cases and controls matched for age +/- 1 year at CHARM III Visit 1 (baseline) and Visit 3 (follow-up), presented as mean (SD), frequency, and % change

	Cases (n=28)			Controls (n=28)			p*
Variable	Visit 1	Visit 3	% change	Visit 1	Visit 3	% change	
Age yrs	44.7 (2.5)	47.38 (2.8)	6.8 (2.5)	44.8 (2.5)	47.7 (2.6)	6.3 (0.8)	0.43
BMI kg/m^2	30.3 (8.4)	30.1 (8.1)	0.04 (6.96)	28.6 (5.0)	28.6 (5.2)	-0.74 (7.05)	0.93
Waist/Hip Ratio	0.82 (0.07)	0.83 (0.09)	1.09 (7.19)	0.80 (0.07)	0.81 (0.06)	0.54 (5.89)	0.63
HOMA-IR	4.1 (2.9)	5.1 (6.0)	42.5 (142.4)	2.9 (1.5)	2.6 (1.5)	1.7 (45.7)	0.28
LH IU/L	7.4 (7.6)	19.4 (26.4)	389 (1030)	7.9 (8.4)	14.7 (16.7)	175 (530)	0.46
FSH IU/L	8.1 (12.7)	18.2 (23.7)	293 (620)	11.7 (14.2)	22.8 (29.3)	189 (513)	0.38
LH/FSH Ratio	1.10 (0.57)	1.33 (0.94)	28.3 (69.4)	0.95 (1.11)	0.88 (0.41)	48 (134)	0.92
E2 pg/mL	83.2 (46.1)	114.3 (102.1)	68.8 (180.1)	70.0 (39.2)	102.7 (106.1)	76 (194)	0.95
TT ng/dL	28.0 (13.5)	24.7 (13.4)	-8.5 (47.8)	24.9 (7.4)	18.8 (12.0)	-23.2 (51.4)	0.14
SHBG nmol/L	139.1 (115.9)	104.3 (66.7)	-13.4 (56.0)	180.8 (113.2)	122.3 (80.7)	-27.1 (34.4)	0.34
FAI	1.09 (0.97)	1.20 (0.87)	32.2 (77.4)	0.62 (0.35)	0.73 (0.63)	31.9 (115.1)	0.30
FEI	0.32 (0.22)	0.55 (0.48)	142 (242)	0.18 (0.14)	0.39 (0.46)	147 (263)	0.87
FAI/FEI	4.39 (3.32)	3.92 (5.41)	-7.8 (82.1)	4.52 (2.87)	3.09 (2.89)	1.5 (117)	0.46
AMH ng/mL	1.22 (1.11)	0.50 (0.72)	-49.6 (32.5)	0.83 (1.72)	0.60 (1.49)	-17.5 (43.2)	0.004

% change= (V3-V1)/V1*100

BMI-Body Mass Index; HOMA-IR-Homeostatic Model Assessment Insulin Resistance; LH-Luteinizing Hormone; FSH-Follicle Stimulating Hormone; E2-Estradiol; TT-Total Testosterone; SHBG-Sex Hormone Binding Globulin; FAI-Free Androgen Index; FEI-Free Estradiol Index

*Mann-Whitney p value

9.0 FIGURES

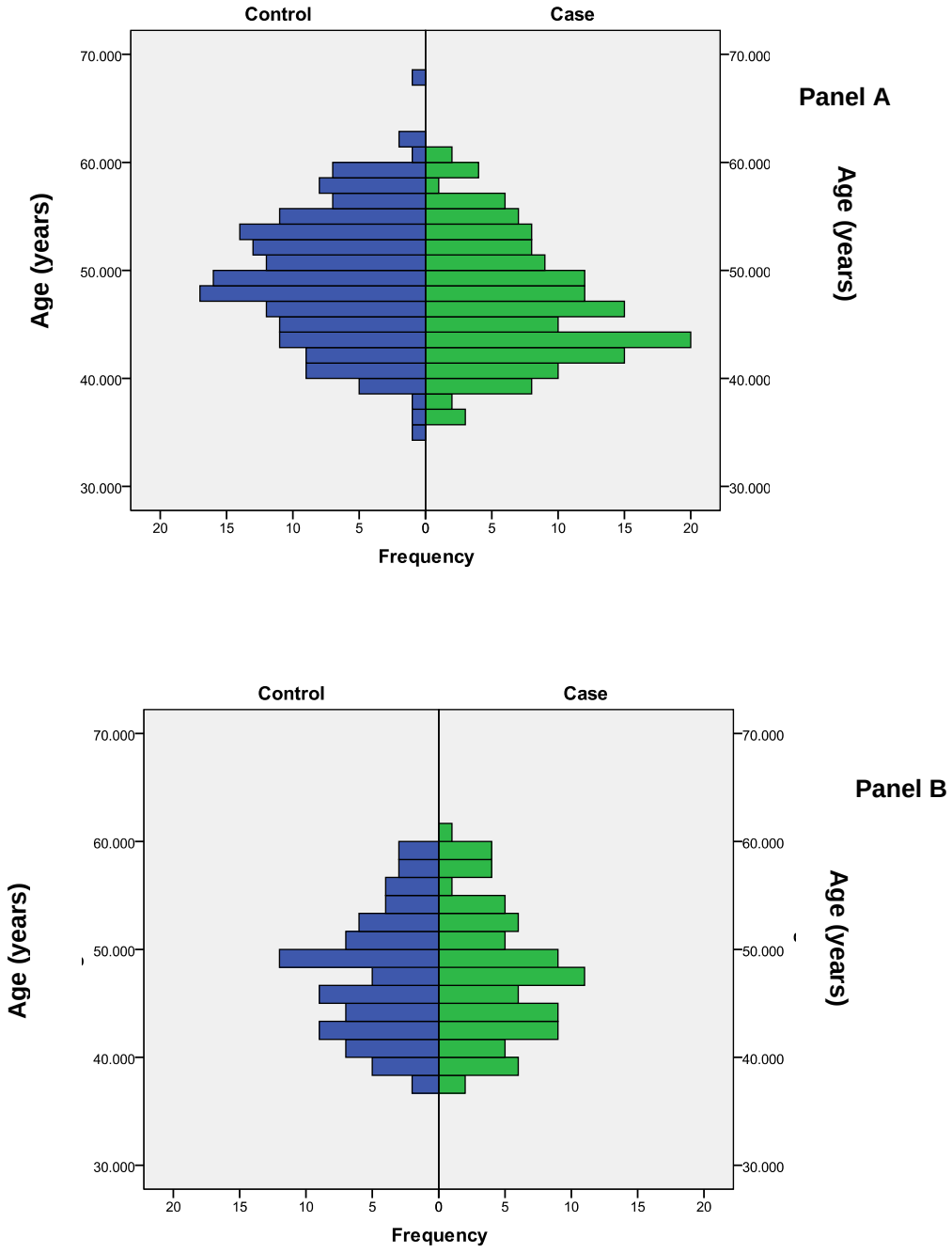
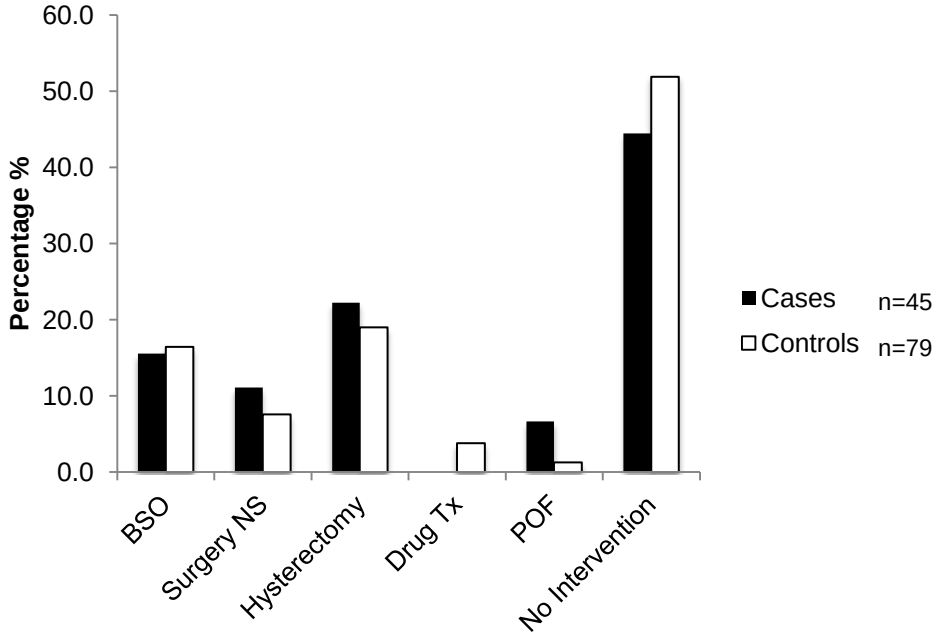


Figure 2. Age distributions of cases and controls at CHARM III visit 1. Panel A (Top) includes all cases (n=152) and controls (n=169). Panel B (bottom) includes 83 case control pairs sequentially matched for age ± one year and ethnicity.



BSO: Bilateral Salpingo-oophorectomy **Surgery NS: Not Specified**
Tx: Drug Treatment **POF: Premature Ovarian Failure**

Figure 3. Reasons menses stopped in cases and controls evaluated at CHARM III visit 1

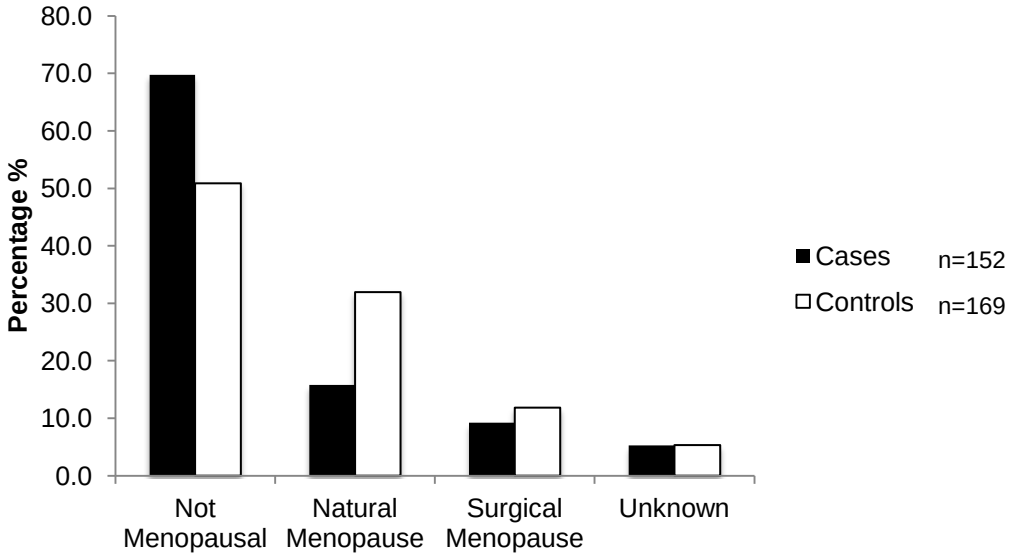
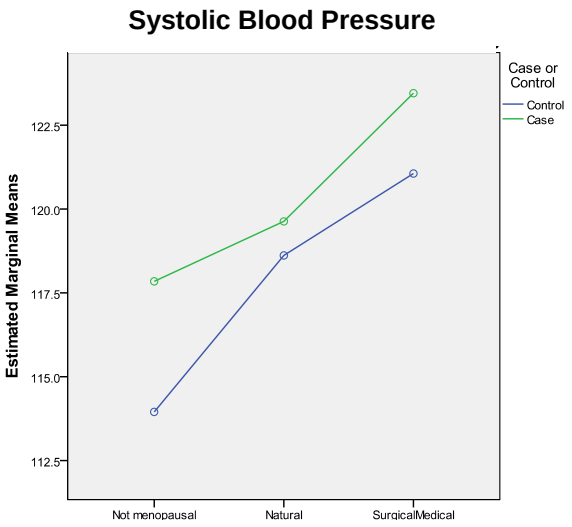
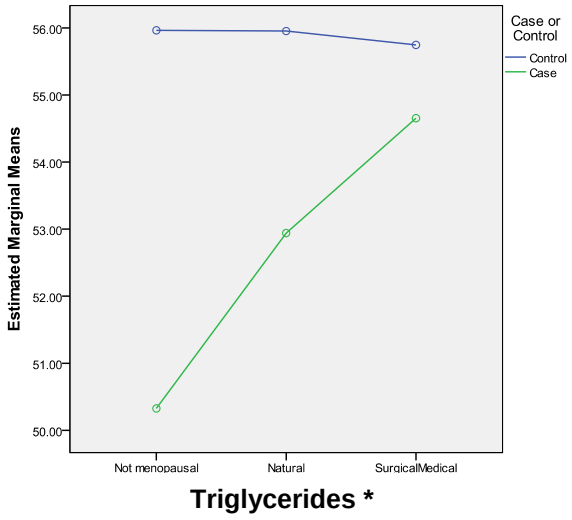


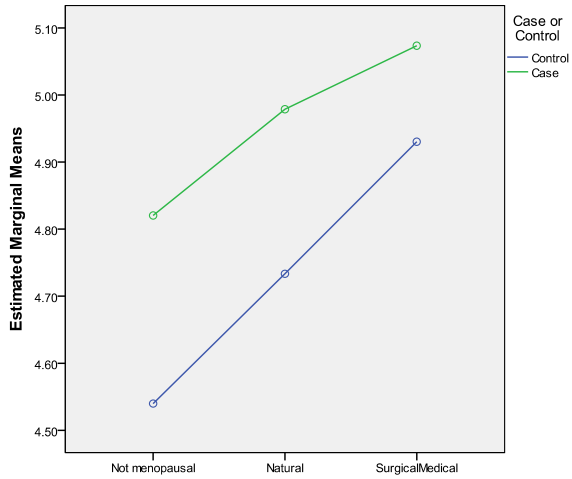
Figure 4. Distribution of reproductive status across cases and controls



Panel A



Panel B



Panel C

Panel A-Systolic Blood Pressure; Panel B-High Density Lipoprotein (HDL); Panel C Triglycerides*-Log transformed

Figure 5. Evaluation of possible interactions on selected cardiovascular disease risk factors in control and PCOS case subjects in CHARM III, visit 1 by reproductive status

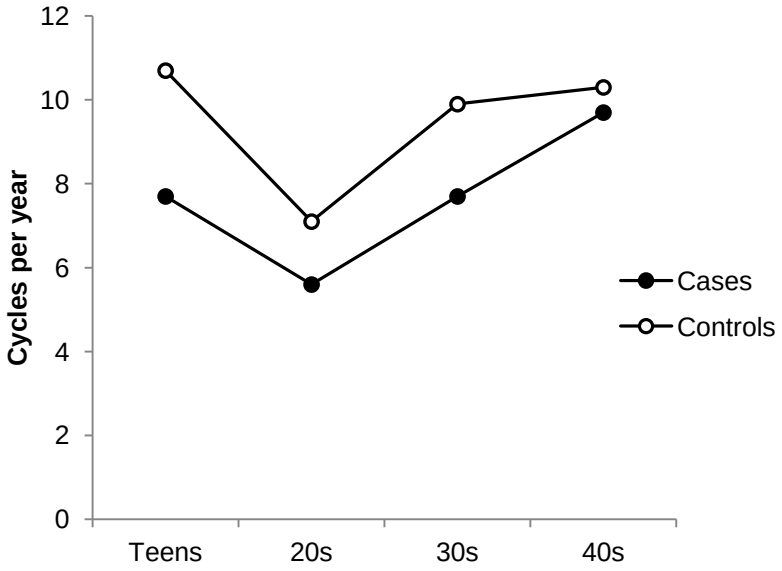


Figure 6. Average number of natural cycles/year at each decade in PCOS cases and controls

APPENDIX A: CHARM III QUESTIONNAIRE

CHARM (Phase III)

Approved Date: October 12, 2011
Renewal Date: October 11, 2012
University of Pittsburgh
Institutional Review Board
Protocol # 0401091

ID# |___|___|___|___|-|___|

WOMEN'S CARDIOVASCULAR HEALTH AND RISK MEASUREMENT STUDY

Clinic Questionnaire
Visit #3

Respondent Name

Last First MI

(If Name Has Changed)

Address

Street

City State ZIP

Phone # Home: (_____) _____ Work: (_____)
Area Area

Social Security # _____/_____/

Date of Birth
Month Day Year

[Let's see, that would make you _____ now.]
Age

Ethnicity _____

Interviewer Initials

Date of Clinic Visit
Month Day Year --------/----------/----------

Visit # 3 ID# |___|___|___|___|___| - |___|

SECTION A: **DEMOGRAPHIC INFORMATION**

A1. What is your current marital status?

1. Married
2. Separated
3. Widowed
4. Divorced
5. Never Married
6. Other (Specify) _____

A2. What is your highest grade or level of schooling completed? (circle number)

Elementary	0	1	2	3	4	5	6	7	8
Secondary	9	10	11	12					
Post Secondary	13	14	15	16	17+				

A2.1 Degree earned

A3. What is your current occupation? _____

A3.1 What category best describes your current job area?

1. Professional
2. Managerial/Administrative
3. Sales
4. Technical
5. Service
6. Clerical
7. Laborer
8. Homemaker
9. Retired
10. Unemployed (Disabled)
11. Unemployed (Not disabled)
12. Other (Specify) _____

A3.2 How many hours do you work per week? _____

Visit #3 **ID#** |___|___|___|___| - |___|

SECTION B: **CURRENT MEDICAL CARE**

B1. Do you have a medical care provider who is responsible currently for your primary health care?

1. Yes

If yes,

Name:

Address:
Street

City _____ State_____ZIP

Phone: (_____)

Treatment:

_____
(medical, surgical, procedure, diagnosis)

B2. Would you like for us to send your study results to your medical care provider (circle)?

1. Yes

Visit #3 ID# |___|___|___|___| - |___|

SECTION C. MEDICAL HISTORY

C1. Have you ever been told by a physician that you have any of the following conditions?

	(a)		(b)	(c)		(d)
	Doctor Diagnosed		Onset Date	Currently Being Treated		Medication
	Yes 1	No 2	Year	Yes 1	No 2	
Diabetes - Insulin Dependent						
Diabetes - NIDDM Mature onset (after age 25)						
Kidney Disease						
Thyroid Disorder						
Hypoactive						
Hyperactive						
Other (Specify _____)						
Ulcer						
Peptic						
Duodenal						
Nervous or Emotional Problem						
Breast Cancer						
Uterine Cancer						
Ovarian Cancer						
Other Cancer Specify _____						
High Blood Pressure						
Angina						
Heart Attack/MI						
Bypass Surgery/Angioplasty						
Circulation Problems						
Stroke						
Arthritis (Specify _____)						
Chronic back pain						

SECTION D: SURGICAL (OPERATION) HISTORY

D1. I would like to ask you about operations or surgical procedures that you may have had during your lifetime. Have you ever had:

	(a) Have you ever had a (type of operation)?			(b) In what month and year did you have this operation?		(c) What was the reason for the operation?
	Yes 1	No 2	Unk 9	Month	Year	Reason
Thyroid Operation						
Gallbladder Operation						
Operation on Your Breast(s)						
Operation to Tie Your Tubes						
Operation to Remove Your Uterus or Ovaries?						
If yes, What Type of Surgery?						
Removal of Uterus Plus Both Ovaries						
Removal of Uterus, But One or More Ovaries Were Not Removed						
Removal of Both Ovaries, But Uterus Was Not Removed						
Removal of One Ovary, But the Uterus and The Other Ovary Was Not Removed						
Wedge resection/"ovarian drilling"						
Other (Specify): _____						
Other (Specify): _____						

Section E: Current Medications

E1. Are you currently taking any of the following prescription medications?

	(a)		(b)		(c)
	Currently Taking Medication?		**Duration of Use**		**Medication Name**
	Yes 1	**No 2**	**Years**	**Months**	
Digitalis					
Diuretics					

1. Thiazide					
2. Non-thiazide					
Coronary Vasodilators					
Antiarrhythmics					
Sedatives					

Barbiturates					
Major tranquilizers (Thorazine)					
Minor tranquilizers (Librium, Valium)					
Antidepressants (Prozac, Zoloft)					
Antihypertensives					

Antidiabetics (Glipizide, Metformin)					
Insulin					
Anticonvulsants (Dilantin)					
Thyroid medications (Synthroid)					
Corticosteroids (Prednisone)					

Anticoagulants (Coumadin)					
Chemotherapy (Methotrexate)					
Antiandrogens (Flutamide)					
GnRH agonists (Leuprolide)					
Other					

Other					

Visit #3 ID# |___|___|___|___|-|___|

E2. Are you taking any over- the- counter (OTC) medications? (Specify) _____ 1. Yes 2. No 9. Unknown

E3. Have you <u>ever</u> used oral contraceptives?

1. Yes (if yes, go to E3a)
2. No (if no, go to E5)
9. Not sure (if not sure, go to E5)

<u>If yes:</u>

E3a. At what age did you start using oral contraceptives? _____ years

E3b. For how long have or did you use oral contraceptives? _____ yrs _____ mos

E4. Are you <u>currently</u> using oral contraceptives?

1. Yes (If yes go to E5)
2. No (If no go to E4a)

<u>If no</u>:

E4a. At what age did you stop using oral contraceptives? _____ years

E5. Have you <u>ever</u> used hormone replacement therapy?

1. Yes (if yes, Go to E5a)
2. No (if no, go to F1)
9. Not sure (if not sure, go to F1)

<u>If yes:</u>

E5a. At what age did you start using hormone replacements? _____ years

E5b. For how long have or did you use hormone replacements? _____yrs _____mos

E6. Are you <u>currently</u> using hormone replacement therapy?

1. Yes (If yes, go to F1)
2. No (if no, go to E5a)
9. Not sure (if not sure, go to F1)

If no:

E6a. At what age did you stop using hormone replacements? _____ years

SECTION F: REPRODUCTIVE AND MENSTRUAL HISTORY

F1. Have you ever been pregnant?

1. Yes (If yes, go to F1a)
2. No (If no go to F2)

F1a. What are the total number of pregnancies you have had?

F1b. What are the total number of live births you have had?

F1c. Please list biological children here with their genders and ages.

	Gender	DOB	Current Age
Child 1			
Child 2			
Child 3			
Child 4			
Child 5			
Child 6			
Child 7			
Child 8			
Child 9			
Child 10			

F2. Have you ever taken any medication to induce ovulation (fertility drugs)? (Such as Clomid, Serophene, Pergonal, Metrodin, hcG)

1. Yes (Please list in table below)
2. No (If no go to F3)

Name of fertility drug	Approx age when taken	Number of cycles taken

The next set of questions are related to your menstrual periods. We are interested in how often and how predictable your cycles have been throughout your life.

F3. How old were you when you had your first menstrual period? _____ years

I will be asking a series of questions related to your menstrual cycles and hormone use throughout several stages of your life, i.e. during your teen years, during your twenties, during your thirties, and so on. In your (fill in the blank) years, ... (ask about hormone use, number and length of cycles, etc.) A check represents a yes response, a dash a negative response.

F4. Lifetime Menstrual /Exogenous Hormone History

	Teens	20s	30s	40s	50s
Hormone Use and Duration (months)					
1. OC					
Duration					
2. Provera					
Duration					
3. HRT					
Duration					
4. None					
Total duration (months)					
When not on hormones or pregnant:					
Avg # periods/year					
Avg cycle length					
1. < 21 days					
2. 22-26 days					
3. 27-32 days					
4. 33-40 days					
5. > 40 days					
6. No periods					

Visit #3 ID# |___|___|___|___|-|___|

F5. Have you had at least one period in the last 12 months?

1. Yes (If yes, go to F6)

2. No (if no, go to F5a)

F5a. How old were you when you stopped having your menstrual period? _____ yrs

F5b. Did your periods stop naturally or because you had an operation, radiation treatment, or some drug therapy?

1. Surgical (Uterus and/or ovaries removed)
2. Natural Menopause
3. Radiation therapy
4. Drug therapy
5. Other _____

F6. What was the first day of your last menstrual period?

Date _____/_____/

F7. Thinking back over the past 12 months, in how many of those months did you have a period?

_____

F8. What would you estimate was the length of your menstrual cycle during the past 12 months?

1. < 21 days
2. 22-26 days
3. 27-32 days
4. 33-40 days
5. >40 days
6. **No periods (postmenopausal?)**

F9. Were you troubled by acne after your teen years?

1. Yes (If yes, go to F9a)
2. No (if no, go to F10)

If yes:

F9a. For how long (in years)? _____ yrs

F9b. Where was (is) the acne located?

1. Face and head
2. Shoulder, back or chest
3. Both 1 and 2

F9c. Do you currently have acne?

1. Yes
2. No

Visit #3 ID# |___|___|___|___|-|___|

F10. Have you ever been troubled by unwanted body hair?

1. Yes (If yes, go to F10a)
2. No (if no, go to Section G)

F10a. Where was this unwanted body hair? (Check all that apply)

upper lip _____
chin
neck
chest
lower stomach
inner upper thighs
sideburns

SECTION G: FAMILY HISTORY

G1. Do you have any family members with Polycystic Ovary Syndrome? This is a condition marked by menstrual irregularity and/or infertility, central body weight gain ("apple shape") and sometimes increased body hair and severe acne.

1. Yes (If yes, go to G2)
2. No (if no, go to G3)
3. Possible (go to G2)
4. Unknown (go to G3)

G2. Which if any of the following first and second degree relatives been diagnosed with PCOS? (List as Y, N, Poss, Unk; note number)

Relative	Diagnosed	No.	Suspected	No.
Maternal Grandmother		NA		NA
Paternal Grandmother		NA		NA
Mother		NA		NA
Sister				
Daughter				

G3. Have any of the following first and second degree relatives developed early baldness, before age 30?

1. Yes (If yes, go to G4)
2. No (if no, go to Section H))
3. Possible (go to G4)
4. Unknown (go to Section H)

G4. Which if any of any of the following first and second degree relatives developed early baldness?
(Y,N,Poss, Unk and note number)

Relative	**Diagnosed** **No.**	**Suspected** **No.**
Maternal Grandfather	NA	NA
Paternal Grandfather	NA	NA
Father	NA	NA
Brother		
Son		

SECTION H: LIFESTYLE DESCRIPTION AND HABITS

H1. Have you ever smoked cigarettes?

1. Yes (If yes, go to H1a)
2. No (if no, go to H3)

If yes:

H1a. At what age did you start smoking cigarettes? _____ Years

H2. Are you currently smoking?

1. Yes (If yes, go to H2b)
2. No (If no, go to H2e)

If yes:

H2b. How many cigarettes, on the average, do you smoke daily?

1. Once in a while, not daily
2. Less than half a pack a day
3. Half a pack up to one pack a day
4. One pack up to two packs a day
5. Two or more packs a day

H2c. For how many years did you or have you smoked? _____ Years

H2d. Has the smoking been continuous? _____Yes _____ No **(Go to H3)**

If no:

H2e. If not smoking currently, how many years did you smoke before you stopped?

_____ Total number of years

_____ Age when stopped

H2f. How many cigarettes, on the average, did you smoke daily at the time you stopped (see below)?

6. Once in a while, not daily
7. Less than half a pack a day
8. Half a pack up to one pack a day
9. One pack up to two packs a day
10. Two or more packs a day

H2g. Has the smoking been continuous? _____Yes _____ No **(Go to H3)**

H3. Do you currently have occasion to drink alcoholic beverages when out socially or relaxing?

1. Yes (If yes, go to H3a)
2. No (if no, go to H4)

If yes:

H3a. On the days that you drink, do you typically drink (circle more than one if necessary):

1. Beer
2. Wine
3. Mixed drinks
4. Hard liquor

H3b. On the days that you drink, how many 8 oz. glasses do you have (on average)? _____ glasses 1) Beer _____ 2) Wine _____ 3) Mixed Drinks _____ 4) Liquor _____

H3c. How often do you drink this amount?

	A.. Drink Type _____	B. Drink Type _____	C. Drink Type _____
1.	Every day	1) Every day	1) Every day
2.	Almost every day	2) Almost every day	2) Almost every day
1.	3-4 times/week	3) 3-4 times/week	3) 3-4 times/week
1.	1-2 times/week	4) 1-2 times/week	4) 1-2 times/week
5.	2-3 times/month	5) 2-3 times/month	5) 2-3 times/month
6.	Once a month	6) Once a month	6) Once a month
7.	6-11 times/year	7) 6-11 times/year	7) 6-11 times/year
8.	1-5 times/year	8) 1-5 times/year	8) 1-5 times/year
9.	< 1 time/year	9) < 1 time/year	9) < 1 time/year

Visit #3 ID# |___|___|___|___| - |___|

Section I: Menopausal Symptoms (Pilot Survey)

I would like to ask you some questions to determine if you are experiencing symptoms related to the menopause.

I1. Are you having any hot flashes, night sweats, or both? ____ Yes _____ No

If yes, please rate:

1) Rarely 2) Sometimes 3) Often 4) Very Often

I2. Do you feel a loss of energy or are more fatigued? ____ Yes _____ No

If yes, please rate:

1) Rarely 2) Sometimes 3) Often 4) Very Often

I3. Are you having insomnia, difficulty falling to sleep, or staying asleep? ____ Yes _____ No

If yes, please rate:

1) Rarely 2) Sometimes 3) Often 4) Very Often

I4. Are you experiencing more times of mental fogginess or having trouble thinking clearly? ____ Yes _____ No

If yes, please rate:

1) Rarely 2) Sometimes 3) Often 4) Very Often

I5. Are you more irritable, have more nervous tension? ____ Yes _____ No

If yes, please rate:

1) Rarely 2) Sometimes 3) Often 4) Very Often

I6. Is your mood low, are you less upbeat, less positive or having more mood swings? ____ Yes _____ No

If yes, please rate:

1) Rarely 2) Sometimes 3) Often 4) Very Often

Visit #3 ID# |___|___|___|___|___| - |___|

SECTION J: **ANTHROPOMETRICS**

J1. Preliminary Measurements:

Bellefield **EBCT**

Weight _____pounds Weigl (withc

Height _____ inches Heigh (withc

Have you lost 10 or more lbs. in the last year?

1. Yes 2. No 9. Unknown

If yes, how many? _____

Have you gained 10 or more lbs. in the past year?

1. Yes 2. No 9. Unknown

If yes, how many? _____

J2. Approximately how much did you weigh (in pounds):

When you graduated from high school / were 18 years old?

When you were 25 years old?

When you were 35 years old?

When you were 45 years old?

When you were 55 years old?

J3. Circumference Measurements (to nearest .1 cm.)

Waist (abdominal) Girth 1) _____ _____ _____ . _____ cm.

Hip Girth 1) _____ _____ _____ . _____ cm.

Visit #3 ID# |___|___|___|___| - |___|

Section H: Standard Pulse and Blood Pressure Assessment

Participants must avoid caffeine for at least 30 minutes prior to assessment. Subject must be quiet and remain in a seated position continuously for 5 minutes prior to and during the 2 measurements. During the measurements of the BP, there should be no change in the position of the participant.

Observers Code: _____ Device Code: _____

H1. Radial Pulse

Beat in 30 seconds _____ X 2 = _____ Beats/minute

Is pulse regular? 1. Yes 2. No

H2. Cuff Size (check one) and Peak inflation level

_____ 1. Regular Adult (16.0 - 22.5 cm) Pulse obliteration pressure (POP) _____
_____ 2. Large Adult (30.1- 37.5 cm) Peak Inflation Level Std man. (PIL) 30
_____ 3. Thigh (37.6- 43.7 cm) Peak Inflation Level (POP + PIL)

	Systolic									Disappearance 5th Phase Diastolic						
Reading 1 (Std)																
Reading 2 (Std)																

Sum of Readings																	
Average																	

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